UNIVERSIDAD AUTÓNOMA DE MADRID

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ESTUDIO DE PÉPTIDOS ALIMENTARIOS CON ACTIVIDADES BIOLÓGICAS RELACIONADAS CON LA FUNCIÓN INTESTINAL

[FOOD PEPTIDES WITH BIOLOGICAL ACTIVITIES RELATED TO INTESTINAL FUNCTIONS]

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INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC-UAM)

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ESTUDIO DE PÉPTIDOS ALIMENTARIOS CON ACTIVIDADES BIOLÓGICAS RELACIONADAS CON LA FUNCIÓN INTESTINAL

Memoria presentada por :

Daniel Martínez Maqueda

Para optar al grado de

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Trabajo realizado bajo la dirección de:

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Instituto de Investigación en Ciencias de Alimentación

ISIDRA RECIO SÁNCHEZ, PROFESORA DE INVESTIGACIÓN DEL CSIC, BLANCA HERNÁNDEZ LEDESMA, CONTRATADA RAMÓN Y CAJAL DEL CSIC, Y BEATRIZ MIRALLES BURAGLIA, TÉCNICO SUPERIOR ESPECIALIZADO DEL CSIC, DEL INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

CERTIFICAN,

Que el presente trabajo titulado "ESTUDIO DE PÉPTIDOS ALIMENTARIOS CON ACTIVIDADES BIOLÓGICAS RELACIONADAS CON LA FUNCIÓN INTESTINAL [FOOD PEPTIDES WITH BIOLOGICAL ACTIVITIES RELATED TO INTESTINAL FUNCTIONS]" y que constituye la Memoria que presenta el Licenciado en Ciencias Químicas DANIEL MARTÍNEZ MAQUEDA, para optar al grado de Doctor con Mención de Doctorado Europeo, ha sido realizado bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación CSIC-UAM.

Y para que conste firmamos el presente certificado a 13 de Mayo de 2013.

Fdo: Isidra Recio Sánchez

Fdo: Blanca Hernández Ledesma

Fdo: Beatriz Miralles Buraglia

A María, mis padres y mi hermano

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Da chimico un giorno avevo il potere

di sposare gli elementi e di farli reagire…

Un chimico (*Fabrizio De André*)

ÍNDICE

ABSTRACT

The gastrointestinal lumen is strategically covered by a protective layer, known as mucus, and its production can be modulated by different food components. β-Casomorphin 7, a casein-derived peptide, has been shown to modify the dynamics of intestinal mucus by increasing the secretion and expression of mucins, the major macromolecular constituent, through an opioid mechanism. In this Thesis, several milk-derived peptides with proved or probable ability to bind μ- and δ-opioid receptors, some of them identified in simulated gastrointestinal digestions, besides two hydrolysates from casein and a whey protein concentrate (WPC) rich in β-lactoglobulin that contain some of the selected peptides, were investigated in HT29-MTX human intestinal goblet-like cells. The WPC hydrolysate exhibited a significant activity with increased mucin secretion and stimulated expression of MUC5AC, the major secreted mucin gene in HT29-MTX cells. This effect was partially supported by the presence in the hydrolysate of the opioid β-lactorphin (YLLF), which demonstrated influence on mucin secretion although not in gene expression. The casein hydrolysate showed a remarkable activity that may result from the α_{s1}-casein fragments 143-149 (AYFYPEL) and 144-149 (YFYPEL), although both peptides induced a lower effect separately. Therefore, other peptides or components of the hydrolysates could participate in the observed activities. In order to elucidate if peptides with effect on mucin production, but not reported opioid activity, could bind opioid receptors, experiments with guinea-pig ileum preparations were performed. Interestingly, α_{s1}-casein fragments 144-149 (YFYPEL) and 144-148 (YFYPE) showed opioid activity for the first time. However, YFYPE exhibited no significant effect on MUC5AC expression, so the mediation of additional pathways should be considered. Mucus strengthening also plays an important role in the gastric mucosa defence. Thus, antiulcerative properties of the WPC and casein hydrolysates were evaluated in an ethanol-induced ulcer model in rats. Both hydrolysates showed effective gastric protection against the induced lesions.

The antiulcerative activity of the WPC hydrolysate was mediated by the action of sulfhydryl (SH) groups, as revealed the lack of protective effect after *in vivo* SH blockage by N-ethylmaleimide. Moreover, diet supplementation with whey proteins is currently considered an alternative and promising treatment of type 2 diabetes, in part due to the observed inhibition of dipeptidyl peptidase IV enzyme (DPP-IV). β-lactoglobulin was evaluated as source of DPP-IV inhibitory peptides. The WPC hydrolysate showed an important inhibition of DPP-IV activity and two β-lactoglobulin fragments, 78-82 (IPAVF) and 78-83 (IPAVFK), were identified for the first time as potent DPP-IV inhibitory peptides, especially IPAVF with an IC_{50} of 44.7 μM. In conclusion, milk protein hydrolysates and derived peptides could be promising for the development of functional foods to improve epithelium protection in gastrointestinal diseases and the management of type 2 diabetes.

RESUMEN

El epitelio gastrointestinal está protegido por una capa, denominada mucus, cuya producción puede estar regulada por diferentes componentes alimentarios. Un péptido derivado de la caseína, la β-casomorfina 7, ha demostrado influencia en la producción del mucus intestinal a través de la estimulación de la secreción y la expresión génica de las mucinas, principales constituyentes macromoleculares, mediante un mecanismo opioide. En esta Tesis Doctoral, se estudió el efecto sobre la producción de mucinas, en células caliciformes intestinales humanas HT29-MTX, de distintos péptidos alimentarios con probada o probable afinidad hacia receptores μ y δ-opioides, algunos de ellos identificados en simulaciones gastrointestinales. Adicionalmente, se ensayaron un hidrolizado obtenido a partir de un concentrado de proteínas de suero (WPC) enriquecido en β-lactoglobulina y un hidrolizado de caseínas y, que contenían algunos de los péptidos seleccionados. El hidrolizado de WPC mostró una actividad significativa sobre la producción de mucinas, incrementando su secreción y sobreexpresando MUC5AC, el gen de la principal mucina secretada en las células HT29-MTX. Este efecto fue parcialmente justificado por la presencia en el hidrolizado del péptido opioide β-lactorfina (YLLF), el cual estimuló la secreción de mucinas pero no modificó la expresión del gen MUC5AC. El hidrolizado de caseínas mostró una potente actividad estimulante sobre la producción de mucinas, que puede provenir de los fragmentos 143-149 (AYFYPEL) y 144-149 (YFYPEL) de la α_{s1}-caseína, aunque ambos péptidos inducían un menor efecto cuando se ensayaron separadamente. Por tanto, otros péptidos o componentes de los hidrolizados podrían participar en sus actividades observadas sobre la síntesis de mucinas. Con el objetivo de elucidar si los péptidos con efecto estimulante sobre la producción de mucinas, pero sin actividad opioide descrita, podrían interaccionar con los receptores opioides, se llevaron a cabo experimentos con íleon de cobayo. Como resultado se demostró, por primera vez, que los fragmentos 144-149 (YFYPEL) y 144-148 (YFYPE) de la α_{s1}-caseína presentan

actividad opioide. No obstante, el péptido YFYPE no provocó sobreexpresión de MUC5AC en las células HT29-MTX, por lo que podría considerarse la mediación de mecanismos adicionales. El fortalecimiento del mucus también desempeña un papel crucial en la defensa de la mucosa gástrica. Por ello, se evaluaron las propiedades antiulcerogénicas de los hidrolizados de caseína y WPC en un modelo de úlceras inducidas por etanol en ratas. Ambos hidrolizados exhibieron una eficaz protección frente a las lesiones gástricas inducidas por etanol. La actividad antiulcerogénica del hidrolizado de WPC se encontró mediada por la acción de los grupos sulfhidrilos (SH) activos, ya que disminuía el efecto del hidrolizado al tratar el animal con el agente bloqueante de los grupos sulfidrilo N-etilmaleimida. Por otra parte, se está estudiando el suplemento de la dieta con proteínas de suero para el control de la diabetes tipo 2, cuya actividad podría estar mediada por la inhibición de la enzima dipeptidil-peptidasa (DPP-IV). Por ello, se evaluó la β-lactoglobulina como una fuente de péptidos con capacidad inhibitoria de esta enzima. El hidrolizado de WPC enriquecido en β-lactoglobulina produjo una importante inhibición de la actividad de la DPP-IV y se identificaron por primera vez dos secuencias de la β-lactoglobulina, concretamente los fragmentos 78-82 (IPAVF) y 78-83 (IPAVFK), como potentes inhibidores de la enzima DPP-IV. El péptido IPAVF demostró una notable actividad inhibitoria, con un valor de IC_{50} de 44.7 μM. En conclusión, dos hidrolizados de proteínas lácteas y una serie de péptidos derivados podrían ser prometedores en el desarrollo de alimentos funcionales para la mejora de la protección del epitelio en enfermedades gastrointestinales y para el control de la diabetes tipo 2.

LISTA DE ABREVIATURAS

ARNm: ARN mensajero

CPPs: fosfopéptidos de caseína

DAMGO: encefalina D-Ala(2),N-Me-Phe(4),glicinol(5)

DPP-IV: enzima dipeptidil peptidasa-IV

ECA: enzima convertidora de angiotensina

GIP: péptido insunilotrópico dependiente de la glucosa

GLP-1: péptido similar al glucagón tipo 1

GMP: caseínmacropéptido o glicomacropéptido

HPLC-MS/MS: cromatografía de alta resolución acoplada a espectrometría de masas en tándem

ILU: índice de lesiones ulcerativas

miARN: micro ARN

Mucn: gen correspondiente a la mucina de rata número “n”

MUCn: gen correspondiente a la mucina humana número “n”

NEM: N-etilmaleimida NEM

NO: óxido nítrico

PPARs: receptores de proliferadores peroxisomales

RN: receptores nucleares

SH: sulfhidrilos

SHR: ratas espontáneamente hipertensas

WPC: concentrado de proteínas de suero

WPI: aislado de proteínas de suero

OBJETIVOS Y PLAN DE TRABAJO

La industria alimentaria está especialmente interesada en el desarrollo de nuevos ingredientes funcionales, que incluyan péptidos y/o hidrolizados proteicos con demostrado efecto beneficioso sobre procesos que ocurren a nivel gastrointestinal, como pueden ser la protección de la mucosa o la secreción de incretinas.

Objetivo general 1

En los últimos años, se ha incrementado el interés por péptidos alimentarios con efectos fisiológicos a nivel gastrointestinal (Moughan et al., 2007; Shimizu & Son, 2007). El epitelio del tracto gastrointestinal constituye la mayor superficie de contacto entre el medio externo y el organismo, estando continuamente expuesto a diversas agresiones, como patógenos y compuestos nocivos que pueden acompañar a los alimentos, así como a las propias condiciones del tracto gastrointestinal (cambios de pH y actividad proteolítica). Especialmente bajo algunas situaciones patológicas como las enfermedades inflamatorias intestinales o las úlceras pépticas, el desempeño de las funciones de las células epiteliales puede verse negativamente afectado. En este contexto, se ha postulado que la fortificación del mucus gastrointestinal, en particular por el efecto de nutrientes, podría ser muy beneficiosa en la protección del epitelio. Un péptido lácteo con actividad opioide, β-casomorfina 7, había demostrado contribuir significativamente a la producción de mucinas, los principales componentes macromoleculares del mucus, mediante la activación de receptores opioides tipo μ en cultivos de células caliciformes intestinales (Zoghbi et al, 2006). Éste y otros péptidos con actividad opioide han sido identificados tras la ingesta de leche (Svedberg et al., 1985), además de poder liberarse tras procesos de hidrólisis enzimática de las caseínas y las proteínas de suero (Teschemacher et al., 2003).

El **objetivo general 1** propone la **búsqueda de péptidos e hidrolizados proteicos de origen alimentario con efecto protector sobre el epitelio**

gastrointestinal a través del incremento de la producción de mucinas por las células caliciformes. Para llevar a cabo este objetivo general se abordan los siguientes objetivos parciales:

- ► Evaluación del efecto de péptidos sintéticos sobre la producción de mucinas en las células caliciformes intestinales humanas HT29-MTX.
- ► Evaluación del efecto sobre la producción de mucinas en células HT29-MTX por parte de hidrolizados proteicos que contienen péptidos con estructuras afines para la interacción con receptores opioides.
- ► Elucidación del mecanismo opioide en la actividad de péptidos sobre la producción de mucinas en células caliciformes intestinales HT29-MTX.
- ► Evaluación de la actividad antiulcerogénica *in vivo* de hidrolizados con capacidad para favorecer la producción de mucinas.

Para conseguir dichos objetivos se sigue el siguiente plan de trabajo:

1. Síntesis mediante química en fase sólida Fmoc de péptidos de origen alimentario con actividad μ- o δ-opioide descrita o cuya secuencia de aminoácidos favorezca la interacción con los receptores opioides.
2. Estudio preliminar de la actividad estimulatoria sobre la secreción de mucinas por parte de los péptidos sintéticos, medida en un cultivo de células intestinales humanas con demostrada capacidad mucosecretora (HT29-MTX). Análisis de las mucinas secretadas mediante un ensayo de lectina asociada a enzimas (ELLA), que determina la cantidad de glicoproteínas de alto peso molecular afines a las mucinas (Trompette et al., 2004).
3. Estudio del efecto sobre la expresión del gen de la mucina 5AC (MUC5AC), la principal mucina secretada en células HT29-MTX, por parte de aquellos péptidos que previamente demuestren actividad sobre la secreción de mucinas. Extracción del ARN total de las células y determinación de los niveles relativos

de ARN mensajero mediante PCR cuantitativa con los oligos descritos por Zoghbi et al. (2006).

4. Desarrollo de hidrolizados proteicos de grado alimentario que contengan los péptidos que previamente hubieran mostrado efecto estimulatorio sobre la producción de mucinas. Cuantificación de los péptidos de interés mediante HPLC-MS/MS (Contreras et al., 2009).
5. Estudio del efecto de los hidrolizados proteicos sobre la secreción de mucinas y expresión de MUC5AC.
6. Estudio de la influencia del mecanismo opioide en la expresión de mucinas, ensayando conjuntamente péptidos activos junto a un antagonista μ-opioide, la ciprodima, en células HT29-MTX.
7. Determinación de la actividad opioide de péptidos con estructura favorable mediante el empleo de preparaciones de músculo longitudinal-plexo mientérico de íleon de cobayo.
8. Determinación de la actividad antiulcerogénica de hidrolizados proteicos de grado alimentario con propiedades estimulatorias en la producción de mucinas en células caliciformes intestinales, empleando un modelo de úlceras inducidas por etanol absoluto en ratas Wistar.
9. Estudio de la contribución de grupos sulfhidrilos activos en las propiedades antiulcerogénicas observadas en los hidrolizados proteicos.

Objetivo general 2

La diabetes tipo 2 está considerada como una de las epidemias del siglo XXI, con una incidencia creciente y unos altos costes sanitarios (Pratley & Salsali, 2007). Afortunadamente, existen tratamientos orales que muestran un efecto prometedor frente a esta enfermedad, como es la administración de inhibidores de la enzima dipeptidil peptidasa IV (DPP-IV) (Namba et al., 2013). La enzima DPP-IV actúa degradando las incretinas, hormonas mayoritariamente responsables de la síntesis de

la insulina en las células pancreáticas β (Holst & Deacon, 2004). El suplemento de la dieta con proteínas de suero está actualmente considerado como una alternativa prometedora en el control de la diabetes tipo 2, en parte justificada por la actividad inhibidora de la DPP-IV de los péptidos liberados tras su digestión (Gunnarsson et al., 2006; Sousa et al, 2012).

El **objetivo general 2** persigue **la evaluación de la capacidad de la β-lactoglobulina como fuente de péptidos con actividad inhibidora de la enzima DPP-IV**. Para ello se sigue el siguiente plan de trabajo:

1. Preparación de un hidrolizado tríptico de grado alimentario de un concentrado de proteína de suero enriquecido en β-lactoglobulina.
2. Determinación de la actividad inhibidora de la DPP-IV del hidrolizado proteico mediante el método espectrofotométrico, con p-Gly-Pro-p-nitroanilina como sustrato cromogénico y diprotina A como control positivo (Tulipano et al., 2011).
3. Fraccionamiento del hidrolizado mediante HPLC a escala semipreparativa y determinación de la actividad inhibidora de la DPP-IV de las distintas fracciones del hidrolizado.
4. Caracterización mediante HPLC-MS/MS de las fracciones más activas.
5. Síntesis química de los péptidos potencialmente activos y confirmación de su actividad inhibidora de la DPP-IV.

1. INTRODUCCIÓN

1.1. Actividad biológica de péptidos y proteínas alimentarias

1.1.1. Visión general

Las proteínas constituyen una extensa familia de macromoléculas que se presenta ampliamente en los alimentos, tanto de procedencia animal como vegetal. Forman parte indispensable de la dieta como fuente de nitrógeno y aminoácidos esenciales, a la vez que pueden llegar a ser fuente de energía como lo son los lípidos y los hidratos de carbono. Alternativamente a estas funciones, las proteínas alimentarias pueden liberar péptidos bioactivos, es decir, péptidos con la capacidad de llevar a cabo alguna actividad fisiológica en el organismo. Una buena parte de las funciones del organismo están mediadas por péptidos endógenos, como pueden ser hormonas, neurotransmisores o antibióticos. Se ha comprobado que muchos de los péptidos bioactivos de origen alimentario pueden compartir cierta similitud estructural con péptidos endógenos, lo que les permitiría interaccionar con receptores en el organismo y desarrollar una determinada actividad biológica (Teschemacher, 2003). Estas secuencias se encuentran inactivas en la secuencia proteica pero pasan a poseer dicha funcionalidad al ser generados *in vivo* por la hidrólisis enzimática propia de la digestión gastrointestinal. La producción de péptidos bioactivos es igualmente posible *in vitro* mediante procesos de hidrólisis con distintas enzimas o fermentaciones con cultivos iniciadores, así como también se pueden generar por reacciones presentes durante el almacenamiento de los alimentos (Korhonen & Pihlanto, 2006). Para que estos péptidos puedan ejercer una determinada función biológica, es crucial que estos alcancen su punto de acción, tanto a nivel local como sistémico, y lo hagan en la forma activa.

Hasta la fecha gran parte de los péptidos bioactivos descritos y comercializados proceden de proteínas lácteas, aunque existe un creciente número de péptidos bioactivos derivados de otras fuentes proteicas alimentarias como son pescados,

carnes y subproductos de su procesamiento industrial (colágeno y gelatina resultante de huesos, piel, tendones o espinas), además de huevo y fuentes vegetales, incluyendo principalmente cereales y legumbres (Rutherfurd-Markwick, 2012). Los péptidos bioactivos pueden actuar sobre los principales sistemas corporales, como son el cardiovascular, digestivo, endocrino, inmunológico y nervioso, habiéndose descrito para ellos una amplia variedad de actividades fisiológicas asociadas, como son antimicrobiana, antihipertensiva, hipocolesterolémica, antitrombótica, antioxidante, favorecedora de la absorción y biodisponibilidad de minerales, inmunomoduladora, opioide y antiproliferativa, entre otras (Miguel et al., 2012). En la **Figura 1**, se puede observar una distribución de las principales funciones biológicas asignadas a péptidos bioactivos. La amplia variedad de actividades biológicas descritas ha llegado a recogerse eficazmente en diferentes bases de datos de péptidos bioactivos como la denominada BIOPEP, permitiendo además el análisis *in silico* de la posible actividad de fragmentos proteicos (Minkiewicz et al., 2008). El potencial de los péptidos bioactivos en la promoción de la salud y el tratamiento de las enfermedades crónicas ha despertado interés científico y comercial a lo largo de la última década.

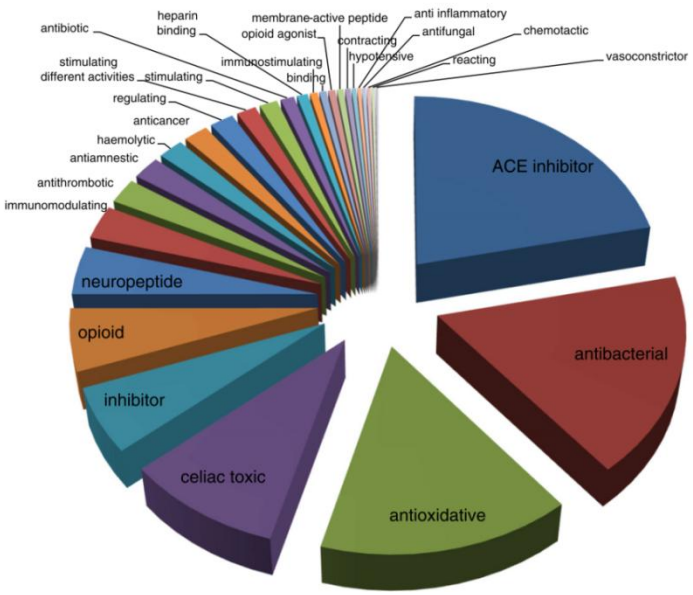

Figura 1: Clasificación de los péptidos bioactivos conocidos en base a su actividad recogida en la base de datos BioPep (n=2594) (Figura tomada de Panchaud et al., 2012).

1.1.2. *Péptidos que modifican la expresión génica. Nutrigenómica.*

La nutrigenómica investiga el efecto de los nutrientes en el organismo, mediante una monitorización de las modificaciones generadas en los patrones de expresión génica (transcriptómica). La nutrigenómica constituye una metodología aplicable a la valoración del efecto beneficioso de los alimentos funcionales, tal como ya se estableció en Japón en 2002 (Nakai et al., 2011). Hay que aclarar que aunque la nutrigenómica comparte con la nutrigenética su objetivo principal basado en el estudio de las interacciones entre la nutrición y la genética, ambas áreas presentan importantes diferencias en sus enfoques. La nutrigenómica se centra en las diferencias de respuesta de distintos nutrientes frente a la expresión génica, mientras que la nutrigenética abarca el estudio de las diferencias entre individuos en relación con su respuesta a un nutriente o pauta dietética en particular (Müller & Kersten, 2003). La nutrigenómica se insertará en el marco de la investigación nutricional orientada a encontrar la mejor "recomendación" dietética entre una serie de alternativas nutricionales, mientras que la nutrigenética será de ayuda en el ámbito de la clínica o de la salud pública a la hora de proporcionar el mejor consejo dietético para un individuo en particular (Carmena & Ordovás, 2005).

Numerosos estudios han demostrado que ciertos nutrientes pueden alterar la expresión génica (Siddique et al., 2009; García-Cañas et al., 2010). Dicha actividad puede desarrollarse a distintos niveles como pueden ser la actuación sobre los factores de transcripción, la generación de cambios epigenéticos o la silenciación génica mediada por micro ARNs (miARN), un tipo de ARN no codificante. Los factores de transcripción son proteínas específicas que interactúan con los nutrientes o sus derivados y que van a regular la expresión génica a través de su unión a regiones concretas del ADN y/o promoviendo modificaciones post-transcripcionales o post-traduccionales. Estas proteínas, también denominadas receptores nucleares (RN), han sido asociadas con diversos ligandos, como son glucocorticoides, hormonas tiroideas,

y otras familias de metabolitos, aunque para muchos de estos receptores no se conoce aún ligando alguno. Un RN típico contiene un dominio de interacción con el ADN, otro para interaccionar con un ligando y otros para interaccionar con activadores funcionales de la transcripción. Para los receptores de proliferadores peroxisomales (PPARs), unos de los RN más estudiados hasta el momento, se han descrito interacciones con carbohidratos, lípidos y algunos aminoácidos, relacionándose con el metabolismo de los lípidos (Cavalieri et al., 2009). Se han descrito otros casos de interacción de nutrientes con RN en la bibliografía (Sanhueza & Valenzuela, 2012).

Los cambios epigenéticos consisten en modificaciones del ADN, que no implican la variación de su secuencia de nucleótidos en sí, pero que van a influir en la expresión génica. La epigenética de un individuo puede ser incluso heredada pero siempre se va a caracterizar por ser potencialmente reversible ante fármacos o nutrientes epigenéticamente activos. En la regulación de la expresión génica mediante cambios epigenéticos se conocen principalmente dos mecanismos: modificación de las histonas y metilación del ADN (Szarc vel Szic, et al., 2010).

Numerosos ejemplos de nutrientes epigenéticamente activos se encuentran en la bibliografía reciente (De Lorenzo, 2012; Ruemmele & Garnier-Lengliné, 2012). Algunos polifenoles como son la genisteína de la soja y la epigalocatequina del té verde son inhibidores de las ADN metiltransferasas (enzimas que catalizan la metilación del ADN), mientras que otros como el resveratrol (antioxidante del vino tinto) y los isotiocianatos (presentes en brócoli, las coles de Bruselas o la coliflor) inhiben la actividad de las histonas deacetilasas (modificadores de histonas). Estos efectos podrían explicar la clara asociación observada entre consumo de fruta y verdura y la disminución del riesgo de padecer varios tipos de cáncer, seguramente evitando la inactivación de genes supresores de tumores. La lunasina, un péptido de 43 aminoácidos (SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD) inicialmente identificado en soja, aunque también presente en cebada, trigo y otras

plantas, se ha mostrado epigenéticamente activo (Hernádez-Ledesma et al., 2011a). En cultivos de células cancerígenas de mama este péptido ha demostrado inhibir la acetilación de las histonas H3 y H4, la cual se asocia con la aparición de procesos tumorales. Esta actividad de la lunasina podría contribuir en sus propiedades quimiopreventivas previamente observadas (Galvez et al., 2001).

Los miARN son secuencias cortas de ARN no codificantes, con un tamaño medio de 19-24 pares de bases. Se estima que regulan más del 60% de los genes codificantes de proteínas, mediante dos principales mecanismos: la degradación del ARN mensajero (ARNm) o la inhibición del inicio de la traducción a partir de estos. Se ha comprobado que mientras algunos miARN sólo regulan procesos individuales específicos, otros pueden llegar a regular la expresión de cientos de genes simultáneamente (Esteller, 2011). Tanto la ginesteína de la soja como el ácido retinoico, presente en distintas verduras, han demostrado tener un efecto anticancerígeno mediado por influencia sobre los niveles de miARN, la primera aumentando los niveles y el segundo disminuyendo los niveles de miARN relacionados con un aumento de procesos tumorales (Banerjee et al., 2008; Weiss et al., 2010). Se ha publicado también el efecto de los folatos sobre la disminución de miARN implicados en la generación de células de la línea leucémica (Sanhueza & Valenzuela, 2012).

El efecto de las proteínas y/o péptidos bioactivos sobre la expresión génica no ha sido tan ampliamente estudiado si se comparan con otros grupos de nutrientes como pueden ser los polifenoles o los lípidos. Las proteínas de la dieta ejercen principalmente su efecto a través de los aminoácidos libres generados, los cuales pueden regular la expresión génica de muchos genes por sí mismos (Brasse-Lagnel et al., 2009). Existen evidencias de que ante una dieta deficitaria en proteínas, la disponibilidad de los propios aminoácidos regula la expresión de numerosos genes (Chaveroux et al., 2010). La ausencia de aminoácidos reprime principalmente la

expresión de genes codificadores de proteínas implicadas en procesos metabólicos de lípidos y carbohidratos, mientras que sobreexpresa genes relacionados con el transporte de membrana para aminoácidos. Otros genes afectados son aquellos relacionados con factores de transcripción (ATF3, C/EBPa, c-jun, etc), proteínas ribosomales o implicados en los procesos de traducción. Endo et al. (2002) estudiaron si la expresión génica en el hígado de rata se podría ver afectada al retirar las proteínas de la dieta. Al compararlo con una dieta que contenía 12% de caseína en un tratamiento de una semana, 281 genes se expresaban diferencialmente, de los cuales 97 se sobreexpresaban y 184 se reprimían. Igualmente, se quiso también estudiar la influencia de la procedencia proteica, comparando entre una dieta basada en caseína y otra en gluten. El resultado fue una menor variación en los niveles de transcripción génica, observándose expresión diferencial en sólo 111 genes (61 genes sobreexpresados y 50 reprimidos) frente a los 281 implicados en la ausencia proteica. Se observó que el metabolismo del colesterol era especialmente sensible a la naturaleza de las proteínas así como a su deficiencia, presentando represión en 11 genes al comparar la dieta libre de proteínas con la dieta basada en caseína y sobreexpresión de 15 genes en la dieta basada en gluten frente a la de caseína. A pesar de dichas diferencias, el nivel sérico de colesterol total fue similar tanto en la dieta con gluten como en la dieta libre de proteínas, registrándose valores algo inferiores a los obtenidos en las ratas alimentadas con un 12% de caseínas. Con el fin de profundizar en el efecto hipocolesterolémico asociado a la proteína de soja, Tachibana et al (2005) llevaron a cabo ensayos con microarrays con el fin de comparar la expresión génica, de nuevo a nivel hepático, en ratas alimentadas durante 8 semanas con una dieta basada en un aislado de proteínas de soja frente a otras alimentadas con caseína. El estudio dio como resultado 63 genes sobreexpresados y 57 genes reprimidos en el grupo alimentado con soja frente al grupo alimentado con caseínas, acompañado de niveles inferiores de triglicéridos y colesterol sanguíneos. Una importante parte de esos genes diferencialmente regulados estaban implicados

en el metabolismo de los lípidos. La distinta concentración remanente de isoflavonas o la cantidad de β-conglicina, proteína con actividad descrita frente a la ateroesclerosis, podría justificar la discrepancia en los resultados para otros aislados de proteína de soja en otros estudios similares (Chou et al., 2012). En cuanto al estudio nutrigenómico de péptidos de origen alimentario, existe un menor número de trabajos en la bibliografía. Un caso lo encontramos para los péptidos IPP y VPP, ambos con una demostrada actividad antihipertensiva, cuyo efecto regulatorio sobre la expresión génica fue estudiado mediante microarray en la aorta de ratas espontáneamente hipertensas (SHR). Los resultados mostraron leves cambios de expresión para genes asociados a los procesos de inhibición de la enzima convertidora de angiotensina (ECA), lo que contribuiría al efecto de estos péptidos en la aorta (Yamaguchi et al., 2009).

1.2. Péptidos con actividad en la función intestinal

El intestino es el órgano responsable de la absorción de nutrientes, ejerce funciones de barrera y de reconocimiento de señales y de síntesis de compuestos bioactivos. Las células epiteliales intestinales se encuentran formando una monocapa que cubre toda la superficie del tracto gastrointestinal, y son especialmente importantes en el desempeño de dichas funciones. Una importante característica de estas células, en comparación con otras células del cuerpo, es que siempre están expuestas a altas concentraciones de factores exógenos, como son los alimentos, los productos de su digestión y los microorganismos. Se conoce que las funciones de barrera y reconocimiento de señales a nivel intestinal están reguladas por hormonas y citoquinas, aunque también los componentes alimentarios juegan un papel fundamental en su modulación (Shimizu, 2010).

Se ha demostrado la capacidad de ciertos péptidos de origen alimentario para actuar sobre la función intestinal (Shimizu & Son, 2007). Aunque la afinidad de estos

péptidos por los receptores celulares es relativamente baja, las altas concentraciones alcanzadas en el lumen pueden provocar efectos fisiológicos importantes, especialmente en aquellos casos donde se dan efectos sinérgicos. Muchos de los péptidos que actúan directamente sobre la modulación intestinal presentan actividad opioide, agonista o antagonista (Rutherfurd-Markwick, 2012). Algunas de las actividades descritas sobre la modulación intestinal son el efecto sobre la saciedad, la regulación del vaciado gástrico, la disminución de la motilidad intestinal y el incremento de la capacidad secretora y absortiva del intestino (Moughan et al., 2007). Otro aspecto importante a considerar es la posible capacidad antioxidante de ciertos péptidos para actuar a nivel intestinal, contrarrestando situaciones de estrés oxidativo e inflamación mediada por citoquinas, fenómenos implicados en el desarrollo de enfermedades inflamatorias intestinales (Young et al., 2010). Un ejemplo lo tenemos en el efecto de la carnosina, la cual exhibe actividad antioxidante acompañada también de un efecto sobre la modulación de la respuesta inflamatoria a través de una disminución en la secreción de citoquinas en células Caco-2 (Son et al., 2004). La respuesta inflamatoria en células epiteliales también se puede ver afectada por la presencia de péptidos opioides de origen lácteo (Neudeck et al., 2003), así como por la acción de los fosfopéptidos de caseína (CPPs) (Otani et al., 2003). La glutamina y, concretamente, algunos dipéptidos que contienen dicho aminoácido y aumentan su disponibilidad, han demostrado actividad reparadora en células epiteliales frente a estrés oxidativo, radiación ionizante, microorganismos y compuestos tóxicos (Satoh et al., 2003; Brito et al., 2005). Otro posible campo de actuación es la influencia directa sobre el transporte de nutrientes por parte del epitelio intestinal. En este caso, el efecto puede estar relacionado con una modificación de la permeabilidad intercelular del epitelio o la activación/inhibición de transportadores activos concretos (Shimizu & Son, 2007). Por ejemplo, el dipéptido AQ ha demostrado evitar el descenso en la expresión de las proteínas PepT1, implicadas principalmente en el transporte de di y tripéptidos, en células Caco-2 bajo estrés oxidativo (Alteheld et al., 2005).

Adicionalmente a la actividad de ciertos péptidos sobre la modulación intestinal, también existe otro grupo de péptidos bioactivos que aunque no actúan directamente sobre el epitelio, van a desarrollar funciones biológicas en el intestino. En este sentido, la influencia sobre el transporte y la absorción de nutrientes se destaca como una de las principales actividades. Por ejemplo, los CPPs procedentes de caseína mejoran la biodisponibilidad del calcio y el hierro, al mantener la solubilidad del mismo en el intestino donde los iones libres serían menos solubles debido al pH neutro-básico (Tsuchita et al., 2001; Bouhallad et al., 2002). Las proteínas de soja y ciertos péptidos derivados han demostrado actividad hipocolesterolemiante al inhibir la formación de las micelas lipídicas, limitando así la absorción de colesterol (Nagaoka et al., 1999). La acción antimicrobiana de ciertos péptidos en el propio intestino toma su relevancia del papel protector que pueden presentar frente a infecciones por patógenos. La lactoferricina B, péptido derivado de la digestión péptica de la lactoferrina bovina, es uno de los péptidos con mayor actividad bactericida, demostrando un amplio espectro de acción contra bacterias Gram- negativas y Gram- positiva (Farnaud & Evans, 2003). Otros péptidos de origen alimentario han demostrado también una importante actividad antimicrobiana (Recio & López-Expósito, 2008).

1.2.1. Mucus intestinal y mucinas: papel protector en el epitelio intestinal

El epitelio gastrointestinal constituye la mayor superficie de contacto entre el medio externo y el organismo. En base a ello, debe desarrollar mecanismos de defensa en respuesta a su vulnerabilidad frente a los agentes externos a los que queda expuesto, como son las condiciones de la propia digestión (cambios de pH y actividad proteolítica de las enzimas secretadas), toxinas o patógenos que puedan acompañar a los alimentos (Deplancke & Gaskins, 2001). El mucus, aparte de ejercer de barrera protectora, va a tener un papel importante en la hidratación del epitelio y va a participar indirectamente en la respuesta inmune, en base a interacciones con las inmunoglobulinas (Forstner & Forstner, 1994). El mucus consiste en un gel altamente

hidratado que presenta una bicapa con una distribución diferenciada en base a la zona del tracto gastrointestinal estudiada. La capa interior está íntimamente ligada al epitelio, presenta una estructura estratificada y compacta y suele estar libre de bacterias. En cambio, la capa exterior presenta una estructura más difusa que permite la entrada de bacterias y sirve de hábitat para la microbiota intestinal (Hansson, 2012). La **Figura 2** muestra el espesor, determinado en rata, de cada una de las capas del mucus gastrointestinal, coincidiendo en gran medida la distribución con la existente en humanos (Atuma et al., 2001). El mucus se encuentra en continua renovación y presenta una estructura que tiene relación con el balance entre la capacidad protectora y la absorción de nutrientes. El espesor es mayor en el estómago y colon, donde debe incrementar la protección frente al pH ácido y las bacterias, respectivamente. En el intestino delgado, donde la absorción de nutrientes es alta, no se observa prácticamente la capa interna ya que los movimientos peristálticos junto con la acción de las proteasas hacen que la renovación de mucus sea constante (Ensign et al., 2012).

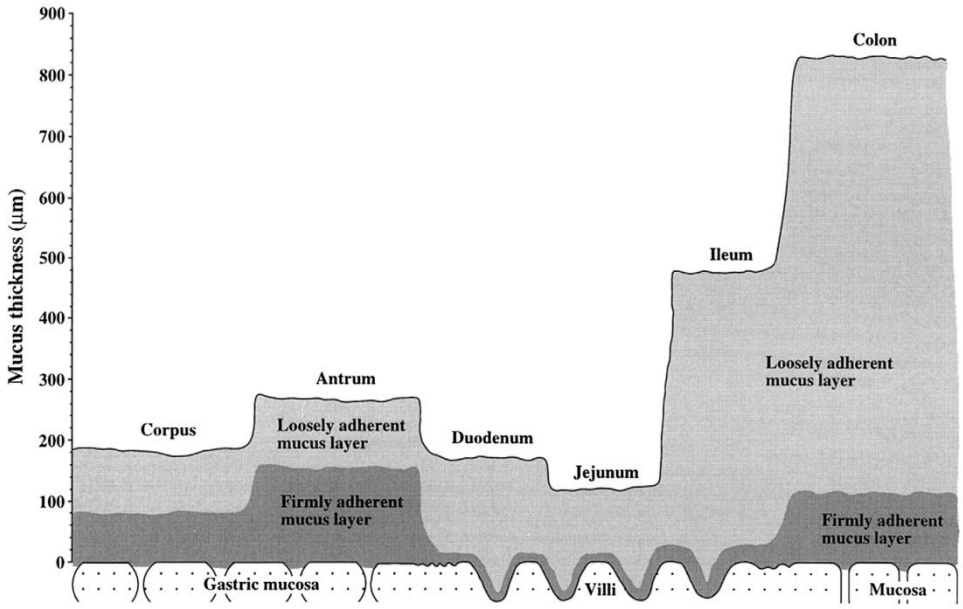

Figura 2: Distribución del mucus a lo largo del tracto gastrointestinal de rata. (Figura tomada de Atuma et al., 2001).

La mejor evidencia de la importancia del mucus intestinal como barrera protectora es la amplia variedad de estrategias usadas por los patógenos para conseguir degradarla o evitarla en su colonización. Éstas incluyen la producción de un amplio número de enzimas degradativas, como glicosulfatasas, sialidasas o mucinasas, y la motilidad a través del gel, siendo ésta última muy importante para su avance, de ahí que muchos microorganismos patógenos sean flagelados (Linden et al., 2008). Al producirse daño en el mucus intestinal por la acción de algún patógeno, estos alcanzan la superficie celular epitelial, produciéndose la infección (**Figura 3**).

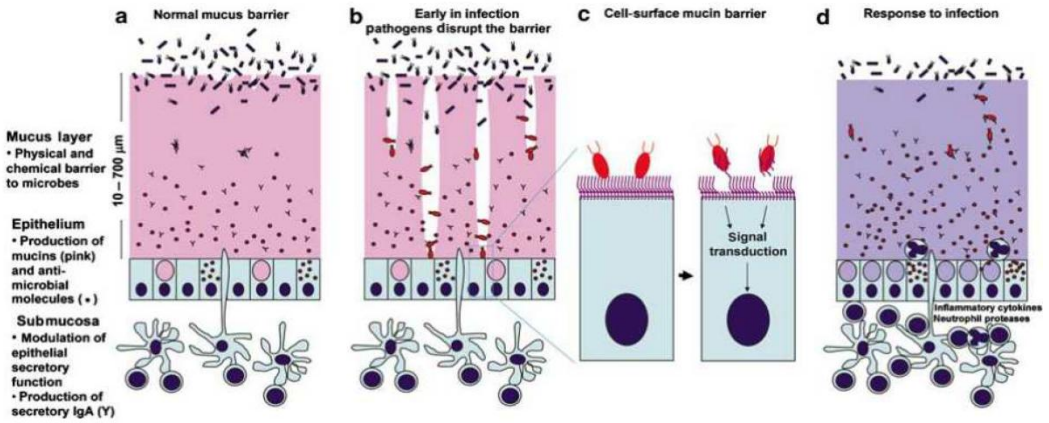

Figura 3: Representación esquemática del papel del mucus en la infección (adaptado de Linden et al., 2008).

Las mucinas constituyen el principal componente macromolecular del mucus intestinal, confiriendo al gel sus propiedades físicas (Juge, 2012). Las mucinas epiteliales representan una familia de glicoproteínas de alto peso molecular que contienen una pronunciada proporción de carbohidratos, comprendiendo más del 70% de su masa total. Se presentan como proteínas ricas en treonina, serina y prolina con una conformación filamentosa, llevando asociada una compleja estructura de oligosacáridos mediantes O-glicosilaciones que les confiere su estructura ramificada (Klein et al., 1993). Hasta la fecha, se han identificado distintas mucinas en el ser humano, observándose que su presencia varía considerablemente a lo largo del tracto

gastrointestinal, tal como se observa en la **Tabla 1**. En cuanto a funcionalidad, las mucinas pueden clasificarse principalmente en dos grupos o categorías: mucinas secretadas y mucinas asociadas a membrana. Las mucinas secretadas constituyen el principal componente macromolecular del mucus, confiriéndole sus propiedades viscoelásticas (Desseyn et al., 2000). En cuanto a composición, las mucinas secretadas son ricas en cisteína en sus dominios N- y C-terminal, lo que favorece la

Tabla 1: Distribución de las mucinas en el tracto gastrointestinal humano (Obtenido de Mackie et al., 2012).

Location	Membrane-associated mucins	Secreted mucins
Oral cavity	MUC1, MUC4, MUC16	MUC5b, MUC7, MUC19
Esophagus	MUC1, MUC4	MUC5b
Stomach	MUC1, MUC16	MUC5ac, MUC6
Small intestine	MUC1, MUC3a, MUC3b, MUC13, MUC17	MUC2
Large intestine	MUC1, MUC12, MUC13, MUC17	MUC2, MUC5b, MUC6

formación de puentes disulfuro intermoleculares (Perez-Vilar et al., 1998). Las mucinas asociadas a membrana se presentan en la superficie apical de las células de los tejidos epiteliales, conteniendo un importante dominio extracelular destinado a la formación de estructuras rígidas. Esto indica que estas mucinas juegan un papel destacado dentro del glicocálix, proporcionando una barrera que evita el acceso hacia la superficie celular de otras células o moléculas de gran tamaño, aunque también se ha sugerido que podrían actuar como sensores frente al contenido luminal (Johansson et al., 2011). Al centrarse en el mucus gastrointestinal, son cuatro las mucinas secretadas a las que se le atribuye la responsabilidad de su formación: MUC2, MUC5AC, MUC5B y MUC6. Como las mucinas en general, éstas presentan un distinto patrón de expresión a lo largo del tracto gastrointestinal. La mucosa estomacal se caracteriza por la liberación de MUC5AC por sus células epiteliales y de MUC6 por las glándulas gástricas. No obstante, el epitelio del intestino delgado y grueso, a través de sus células caliciformes, se destaca en la síntesis de MUC2 (Mackie et al., 2012).

Debido al papel tan importante que juegan las mucinas en las propiedades estructurales del mucus intestinal, cualquier modificación cuantitativa y/o cualitativa, tanto de su secreción como de su expresión, puede afectar a la eficacia protectora del mucus intestinal, pudiendo conllevar algunas implicaciones fisiológicas o patológicas de importancia (Linden et al., 2008). Numerosos estudios apoyan la hipótesis de que alteraciones en la síntesis, secreción o degradación de las mucinas pueden estar implicadas en el comienzo o mantenimiento de enfermedades gastrointestinales (Hansson, 2012). La existencia de una permeabilidad intestinal anormal ha sido implicada en la patogénesis de las enfermedades inflamatorias intestinales, como la colitis ulcerosa o la enfermedad de Crohn (Farhardi et al., 2003). Una mayor permeabilidad facilita el acceso a la mucosa de factores luminales pro-inflamatorios que pueden activar respuestas inflamatorias en cascada. Esta circunstancia, junto a la alteración inmunológica, podrían repercutir en una inflamación incontrolada y daño tisular, fenómenos que se observan en estas enfermedades inflamatorias intestinales. Se han descrito alteraciones en la expresión de la mucina MUC2, la más abundante en el mucus intestinal, en pacientes con enfermedad de Crohn, colitis ulcerosa y neoplasia colónica (Pullan et al., 1994; Byrd & Bresalier, 2004). Como en el ser humano, la mucina secretada mayoritaria en el intestino de los roedores es Muc2. Corroborando la hipótesis del papel protector de las mucinas frente a la infección, existen datos *in vivo* de ratones genéticamente deficientes en Muc2, en los que se ha podido observar el desarrollo espontáneo de inflamación intestinal y posteriormente el de cáncer colorectal (Velcich et al., 2002; Van der Sluis et al., 2006). Con estos estudios queda reforzado el destacado papel que juegan las mucinas en el mantenimiento de la integridad intestinal. Otro interesante estudio ha mostrado el efecto que el estrés psicológico (por inmersión en agua) puede tener sobre la salud gastrointestinal de las ratas objeto de estudio, reduciendo la síntesis de Muc2 y del número de células caliciformes (Shigeshiro et al., 2012).

1.2.2. Péptidos con actividad estimulante de la producción de mucinas

En el contexto en el que el fortalecimiento del mucus intestinal se asume como factor beneficioso en diversas enfermedades inflamatorias intestinales, resulta de gran interés el estudio de la influencia que los componentes de la dieta puedan tener sobre la producción del mucus intestinal. Afortunadamente, ciertos componentes alimentarios han demostrado, tanto en ensayos *in vitro* como *in vivo,* tener una influencia positiva sobre las características del mucus intestinal, aunque el modo de acción de cada compuesto puede diferir (Moughan et al., 2007). Los primeros resultados en este sentido se aportaron en relación a la capacidad de la fibra alimentaria y los ácidos grasos de cadena corta para modificar el mucus intestinal, aumentando la síntesis de mucinas o incluso incrementando el número de células caliciformes responsables de su producción (Barcelo et al., 2000). El efecto de la fibra dietética sobre la producción de mucinas en el lumen del intestino delgado es dependiente de la cantidad y de la calidad de la fibra dietética ingerida, y puede deberse a un incremento en el número de células caliciformes (Tanabe et al., 2005; Ito et al., 2009). En cuanto a los ácidos grasos de cadena corta resultantes principalmente de la degradación por parte de la microbiota intestinal de los carbohidratos no utilizables por el organismo, se ha descrito que el butirato presenta el efecto más importante en la modulación de las mucinas, estimulando la expresión de MUC2 a concentraciones bajas y reprimiéndola a concentraciones elevadas en cultivos de células intestinales humanas (Gaudier et al., 2004; Burger van Paassen et al., 2009). También la administración de ciertos probióticos ha demostrado efecto *in vivo* sobre la expresión de mucinas en el intestino de rata, especialmente incrementando la expresión de Muc3 (Dykstra et al., 2011).

Algunos trabajos se han centrado en estudiar el papel que las proteínas alimentarias, y sus secuencias peptídicas derivadas, pueden jugar en la regulación del mucus intestinal y las mucinas implicadas. Tomando como modelo yeyuno de rata

aislado, se pudo comprobar que dos hidrolizados de α-lactoalbúmina y caseína, junto a una familia de péptidos opioides derivados de la β-caseína, las β-casomorfinas, y en especial el péptido β-casomorfina 7 inducen una potente liberación de mucinas a través de la activación del sistema nervioso entérico y los receptores opioides (Claustre et al., 2002; Trompette et al.., 2003). La presencia de receptores opioides en las células epiteliales intestinales plantea la posibilidad de que las β-casomorfinas, que se producen en el lumen intestinal durante la digestión (Svedberg et al., 1985), podrían regular la producción de mucinas mediante la acción directa sobre las células caliciformes intestinales, no siendo necesarios su absorción y posterior transporte por el torrente sanguíneo. Posteriormente, se valoró directamente el efecto de la β-casomorfina 7 sobre modelos celulares, evitando así la interacción con el sistema nervioso. Dichos modelos están basados en líneas celulares intestinales, tanto humanas (HT29-MTX; Lesuffleur et al., 1993) como de rata (DHE; Trompette et al., 2004), con demostrada capacidad secretora de mucinas y presencia de receptores opioides, con lo que proporcionan una herramienta muy adecuada para el estudio de la regulación de la secreción de las mucinas. Los resultados señalaban el efecto de la β-casomorfina 7 sobre el incremento en la secreción de mucinas y la sobreexpresión de los principales genes implicados en ambos modelos (MUC5AC en HT29-MTX y Muc2 en DHE), estando mediada dicha actividad por receptores μ-opioides. La aplicación de un agonista μ-opioide, la encefalina D-Ala(2),N-Me-Phe(4),glicinol(5) (DAMGO), es capaz de reproducir dichos efectos y el pretratamiento con un antagonista μ-opiáceo, la ciprodima, en cambio, los anula. Todo ello sugiere una actuación directa de la β-casomorfina 7 sobre los propios receptores opioides presentes en dichas líneas celulares y en el propio epitelio intestinal (Zoghbi et al., 2006).

Han et al. (2008) han descrito el efecto *in vivo* que un hidrolizado de caseína tiene sobre la expresión de mucinas en el intestino de rata, observándose

sobreexpresión para los genes Muc3 en el intestino delgado y Muc4 en el colon, tras ser alimentadas con dicho hidrolizado durante 8 días. Por el contrario, una dieta compuesta por aminoácidos libres que emula la composición del hidrolizado, no ejerce ningún efecto sobre la expresión de los genes de mucinas estudiados. En otro estudio, tras administrar un suero de quesería a ratas con inflamación inducida en el intestino grueso, se registró un incremento de la secreción de mucinas pero sin afectar a la expresión del gen Muc2 (Sprong et al., 2010). Los efectos encontrados se atribuían a la contribución de los aminoácidos treonina y cisteína, aminoácidos limitantes en la síntesis de mucinas, principalmente en condiciones de inflamación intestinal. Estos aminoácidos se presentan en una cantidad apreciablemente mayor en las proteínas de suero que en las caseínas. Al administrar una dieta de caseína suplementada en treonina y cisteína, se observó un efecto comparable. Ya previamente, un estudio había mostrado que la suplementación en cisteína, treonina, prolina y serina, aminoácidos muy abundantes en las mucinas, mejora la producción de mucinas a través de un aumento de la secreción, sin cambios en la expresión del gen Muc2 en ratas con inflamación intestinal inducida (Faure et al., 2006). Recientemente, Plaisancié et al. (2013) ha demostrado que el conjunto de péptidos procedentes de una leche fermentada exhibe efecto sobre la producción de mucinas en la línea celular intestinal humana HT29-MTX, mediante el aumento de la secreción y la sobreexpresión de los genes MUC2 y MUC4, sin afectar a MUC5AC, la principal mucina secretada en estas células. De los péptidos contenidos en dicha leche fermentada, el fragmento 94-123 (GVSKVKEAMAPKHKEMPFPKYPVEPFTESQ) de la β-caseína es capaz de emular dicha actividad en el cultivo celular y además mostrar actividad *in vivo* en ratas, favoreciendo la aparición de un mayor número de células caliciformes y una sobreexpresión asociada de los genes Muc2, principal mucina secretada en el intestino, y Muc4.

1.2.3. Péptidos con actividad antiulcerogénica

El estómago se caracteriza por estar expuesto a unas condiciones especialmente agresivas para la digestión, caracterizadas por un pH muy bajo (entre 0,9 y 2,0 en condiciones de ayuno en un adulto sano), resultante de la secreción del acido clorhídrico, y la acción de la pepsina. La mucosa gástrica desempeña un papel crucial en la protección del tejido más interno, ya que una exposición continua a estas condiciones desencadenaría fácilmente un proceso patológico (Melo et al., 1993). Se conoce que la mucosa gástrica posee mecanismos de defensa ante esos agentes nocivos como la presencia de mucus, bicarbonato, prostaglandinas, óxido nítrico (NO) y compuestos con grupos sulfhidrilos (SH) como proteínas, glutatión u otros agentes, así como una adecuada circulación sanguínea que contribuye diluyendo y/o neutralizando los compuestos nocivos, lo que previene su acumulación en la mucosa (Abdel-Salam et al, 2001; Mezzaroba et al., 2006). Junto a la acción protectora del mucus (Niv & Boltin, 2012), el bicarbonato forma en él un gradiente de pH que va desde el propio del contenido gástrico hasta pH 7 en las proximidades de la mucosa gástrica (Allen et al., 1993). La ulcerogénesis péptica, tanto gástrica como duodenal, se produce como resultado de un desequilibrio entre dichos mecanismos de defensa y los agentes nocivos, incluyendo aquellos de naturaleza química o las infecciones por agentes biólogicos, como la bacteria *Helicobacter pylori* (Sachs et al., 2011).

En los casos de úlcera péptica existen diversos tipos de intervención farmacológica. Uno de ellos es la regulación de la secreción de ácido bien mediante el empleo de antagonistas H_2, que actúan bloqueando los receptores de la histamina, un señalizador para la producción de ácido, o bien con inhibidores de la bomba de protones (inhibición de la enzima H+/K+ ATPasa). Es frecuente también el uso de sustancias antiácidas o bases, que actúan neutralizando el ácido generado (Rang et al., 1997). Otro método es la administración de sustancias con grupos SH, ya que actúan como antioxidantes, capturando los radicales libres y protegiendo la integridad

celular. Dentro de las sustancias con grupos SH, parece que el glutatión es el que tiene un mayor efecto, al conjugarse con algunas prostaglandinas (PGE_2 y PGI_2), compuestos relacionados con respuestas inflamatorias y cuya carencia está relacionada con la existencia de erosiones y lesiones gástricas, como ocurre en el caso de la ingesta de fármacos antiinflamatorios no esteroideos (por ejemplo, ácido acetilsalicílico) (Szabo et al., 1992). También algunos medicamentos influyen en el metabolismo de las prostaglandinas, aumentando el nivel de prostaglandinas citoprotectoras (por ejemplo, la carbenoxolona sódica). Por otro lado, la aportación de NO mediante la administración de donadores o la suplementación de arginina, aminoácido precursor del mismo, parece tener un papel protector de la integridad de la mucosa gástrica frente a la agresión por diversos agentes, lo que puede representar una alternativa terapéutica (Barrachina et al., 2001). Por último, en aquellos casos donde se ha producido infección por parte de *Helicobacter pylori*, el tratamiento se basa principalmente en la administración de antibióticos, aunque se está observando una tolerancia progresiva de la bacteria a los mismos y se buscan alternativas terapéuticas complementarias (Sachs et al., 2011).

Paralelamente al desarrollo de los medicamentos empleados en el tratamiento de las úlceras pépticas, hay un creciente interés en torno al uso de sustancias de origen natural, especialmente alimentos, con un efecto complementario (Vismaya et al., 2011). En 1987, Dial & Lichtenberg describieron la actividad antiulcerogénica de la leche de vaca, tanto entera como desnatada, en tres modelos de úlceras inducidas en ratas (0,6 N HCl, etanol absoluto o ácido taurocólico 160 mM). El efecto protector conseguido varía en función del modelo, ya que cada uno de los agentes puede inducir un daño distinto en la mucosa gástrica. Entre las propiedades biológicas asignadas a las proteínas de suero también se encuentra su capacidad antiulcerogénica. Varios estudios han demostrado que la administración de concentrados de proteínas de suero (WPC), hidrolizados de proteínas de suero,

fracciones de dichos hidrolizados o la propia α-lactoalbúmina, pueden proteger a la mucosa gástrica de daños generados por diversos agentes. Este efecto protector se ha explicado en gran parte en base a la intervención de compuestos con grupos SH, principalmente el glutatión, que actúan activando la síntesis de prostaglandinas (Tavares et al., 2011). Matsumoto et al. (2001), describieron la actividad antiulcerogénica de un aislado de proteínas de suero (WPI). Se consideró que la actividad se debía exclusivamente a la presencia de α-lactoalbúmina, demostrando que dicha proteína poseía una gran actividad protectora al ensayarse de manera individual. Posteriormente, se han descrito trabajos sobre la actividad antiulcerogénica de distintos WPC, alcanzando valores muy significativos de protección en úlceras inducidas por etanol (reducción del 73% en el índice de lesiones ulcerativas, ILU, frente al 86,5% recogido para el fármaco carbenoxolona) (Rosaneli et al., 2002). Esa protección fue algo menor aunque no dejó de ser importante (reducción del 50% ILU) en úlceras inducidas por indometacina, antiinflamatorio no esteroideo que inhibe la síntesis de prostaglandinas (Rosaneli et al., 2004). Mezzaroba et al. (2006) volvieron a confirmar la actividad asociada a la α-lactoalbúmina, obteniendo reducciones cercanas al 50% ILU en modelos de úlceras inducidas por etanol e indometacina y empleando dosis cinco veces inferiores a las empleadas anteriormente para el WPC (200 vs 1000 mg/kg rata). Posteriormente, dicho efecto protector de la α-lactoalbúmina se ha justificado en su capacidad para fortalecer el mucus gástrico, por un mecanismo independiente de las prostaglandinas (Ushida et al., 2007). Respecto a los hidrolizados de proteínas de suero, Pacheco et al. (2006) describieron la actividad antiulcerogénica de uno de ellos y de su fracción menor de 1 KDa, mostrando valores mayores que el WPC de partida en tratamientos de una dosis en un modelo de úlcera generada por etanol absoluto (65,5 y 69,3% de reducción ILU para hidrolizado y fracción menor de 1 KDa, respectivamente, frente al 38,2% observado para el WPC de partida). El estudio del mecanismo permitió concluir que la actividad antiulcerogénica mostrada por el hidrolizado y la fracción menor de 1 KDa, depende en buena parte de los compuestos

SH presentes en el hidrolizado. En el caso de la fracción menor de 1 KDa, la estimulación de la síntesis de prostaglandinas endógenas desempeña también un papel importante en la protección gástrica. Recientemente, un concentrado de péptidos y su fracción menor de 3 KDa, resultantes de la hidrólisis de un WPC con proteasas vegetales de *Cynara cardunculus*, mostraron actividad antiulcerogénica en úlceras inducidas por etanol, alcanzando reducciones en ILU de 37,4 y 68,5%, respectivamente, tras dosis únicas de 100 mg/kg rata (Tavares et al., 2011). La actividad mostrada por la fracción menor de 3 KDa está muy influida por la presencia de compuestos SH, mientras que la actividad observada para el concentrado de péptidos parece estar más mediada por la producción de las prostaglandinas y NO. Alternativamente, en úlceras inducidas por etanol, también se ha comprobado el efecto protector del colágeno, tanto de origen bovino como porcino, junto al de un hidrolizado y fracciones del mismo, no observándose diferencias significativas entre hidrolizados y fracciones (Castro et al., 2007). En un estudio posterior se comprobó un efecto sinérgico en la actividad antiulcerogénica al administrar conjuntamente un WPI junto a un hidrolizado de colágeno porcino o bovino. El efecto de los hidrolizados de colágeno está relacionado con un fuerte incremento en la producción de mucus, mientras que la actividad del WPI estaba estrechamente relacionada con los compuestos SH, por lo que la administración conjunta consigue un efecto protector mejorado (Castro et al., 2010).

1.2.4. Péptidos con actividad inhibidora de la enzima dipeptidil peptidasa-IV

La diabetes mellitus es un desorden metabólico considerado como uno de los principales problemas sanitarios a nivel mundial. El número de personas afectadas por esta enfermedad crónica podría alcanzar los 333 millones en 2025, diagnosticándose como tipo 2 aproximadamente un 90-95% de los casos (Pratley & Salsali, 2007). La diabetes tipo 2 se caracteriza por varios defectos patofisiológicos, incluyendo un exceso de producción de glucosa hepática y la progresiva disfunción de las células β

pancreáticas (Bharatam et al., 2007). Se calcula que más del 50% de la insulina total secretada durante la ingesta de alimentos procede de la respuesta de las incretinas, principalmente mediada por los efectos combinados del péptido insunilotrópico dependiente de la glucosa (GIP) y el péptido similar al glucagón tipo 1 (GLP-1) (Girard, 2008). GIP y GLP-1 son secretados en respuesta a la presencia de nutrientes en el lumen intestinal y actúan estimulando la secreción de insulina por parte de las células β pancreáticas (Holst & Deacon, 2004; Mentlein, 2005). Se ha demostrado que el suministro intravenoso de GLP-1 normaliza los niveles de glucosa en enfermos diabéticos aunque los efectos son de corta duración, debido a que más del 95% de esta hormona va a ser rápidamente degradada en la sangre por acción de la enzima dipeptidil peptidasa-IV (DPP-IV) circulante (Drucker, 2006). Debido a esta circunstancia, la administración oral de inhibidores específicos de la DPP-IV se ha presentado como un tratamiento prometedor de la diabetes tipo 2 (Hunziker et al., 2005; McIntosh et al., 2005; Gattrell et al., 2012).

La DPP-IV es una proteasa ampliamente presente en distintos tejidos, incluyendo riñón, hígado, pulmones, epitelio intestinal, linfocitos y células endoteliales. Ejerce su actividad enzimática preferentemente sobre péptidos con prolina o alanina en la segunda posición amino terminal (Thoma et al., 2003). Bastantes neuropéptidos, citoquinas, quimioquinas y hormonas gastrointestinales, incluyendo GIP y GLP-1, constituyen sustratos endógenos de la DPP-IV, por lo que está demostrado el importante papel de esta enzima en múltiples procesos biológicos (Lambeir et al., 2003; Cohen et al., 2004). Mediante experimentos en animales y ensayos clínicos en humanos se ha comprobado que la administración de inhibidores específicos de la DPP-IV repercute en un aumento de la vida media del GLP-1 circulante, un descenso en la glucosa plasmática y una mayor tolerancia a la glucosa (Deacon et al., 1998; Deacon et al., 2000; Mitani et al., 2002). Teniendo en cuenta los altos costes sanitarios asociados a los tratamientos de enfermedades relacionadas con la diabetes tipo 2, el

empleo de distintas estrategias alimentarias ha sido ampliamente estudiado. Por ejemplo, el suplemento de la dieta con proteínas de suero se está estudiando como una alternativa prometedora en la prevención y tratamiento de la diabetes tipo 2 y las enfermedades asociadas, tanto en humanos como en animales (Gunnarsson et al., 2006; Petersen et al., 2009). Se han propuesto algunos mecanismos para explicar el efecto de las proteínas de suero en el tratamiento de dichas enfermedades, entre los que se encuentra el aumento de la tolerancia a la glucosa por parte de los pacientes diabéticos, la reducción del peso corporal o la modulación de hormonas intestinales como la colecistoquinina, leptina y GLP-1 (Sousa et al., 2012).

Adicionalmente a la actividad asociada a las proteínas intactas, hay que tener en cuenta otras funciones fisiológicas que pueden ser atribuidas a los péptidos bioactivos contenidos y liberados durante la digestión o durante el procesado industrial previo (Hernández-Ledesma et al., 2008; 2011b). Recientemente, Lacroix and Li-Chan (2012a) han mostrado resultados *in silico* relacionados con el papel prometedor que las proteínas de suero y las caseínas podrían jugar como precursores de péptidos inhibidores de la DPP-IV tras valorar 34 proteínas de 9 fuentes alimentarias distintas. Resultados obtenidos en estos últimos años han demostrado el efecto inhibitorio de algunos hidrolizados de proteínas de suero, principalmente de β-lactoglobulina, aunque el número de péptidos identificados como potencialmente responsables no es muy alto (Tulipano et al., 2011; Uchida et al., 2011; Nongonierma & Fitzgerald, 2013a; 2013b). Por ejemplo, se ha descrito una apreciable actividad inhibitoria de la DPP-IV para un hidrolizado péptico de proteínas de suero (Lacroix & Li-Chan, 2012b). También se ha identificado un efecto variable para distintos hidrolizados de caseína y lisozima, preparados con distintas enzimas, indicando que la capacidad inhibitoria está muy influenciada por la enzima utilizada (Pieter, 2006; Aart et al., 2009).

La mayoría de los péptidos descritos con actividad inhibitoria tienen una extensión comprendida entre 2 y 7 aminoácidos, caracterizándose por poseer una

naturaleza hidrofóbica y presentar una prolina en la secuencia, preferentemente en la primera o segunda posición amino terminal (Huang et al., 2012). Se conoce que la enzima DPP-IV se une preferentemente a dipéptidos X-P y X-A, aunque péptidos más largos que presentan esas características estructurales también han mostrado que pueden ser sustratos de la enzima. De hecho, los tripéptidos IPI (diprotina A) y VPL (diprotina B), ambos conteniendo prolina en la segunda posición, son sustratos destacados de la enzima y se usan normalmente como referencia (Umezawa et al., 1984). El fragmento 78-80 de la β-lactoglobulina, IPA, con una estructura semejante, ha mostrado una potente actividad inhibitoria de la DPP-IV (Tulipano et al., 2011). También el fragmento 15-20 de la β-lactoglobulina, VAGTWY, ha sido descrito como un importante inhibidor de la DPP-IV (Uchida et al., 2011). En un queso Gouda se identificó un péptido con una significativa actividad inhibitoria de la DPP-IV, concretamente el fragmento 70-77 de la β-caseína (LPQNIPPL). Asímismo, se comprobó su efecto *in vivo* sobre la glucosa plasmática mediante test de tolerancia en un modelo de rata (Uenishi et al., 2012).

1.3. Ingredientes comerciales basados en hidrolizados proteicos

En los últimos años se ha producido una comercialización creciente de alimentos funcionales basados en péptidos bioactivos. Está previsto que una parte importante de las investigaciones descritas en este área se traslade al desarrollo de los correspondientes alimentos funcionales, una vez se vayan optimizando individualmente aspectos como la biodisponibilidad, la producción a escala industrial, las características organolépticas, las interacciones con la matriz alimentaria y la estabilidad de los péptidos bioactivos durante las condiciones de procesado y almacenamiento del alimento (Hernández-Ledesma et al., 2011c). Todos estos factores van a influir considerablemente en el modo en que el alimento funcional vaya a ser comercializado. Muchos hidrolizados proteicos contienen un porcentaje apreciable de péptidos de bajo peso molecular con aminoácidos hidrofóbicos, que

aportan un sabor amargo. Se ha descrito la eliminación del amargor mediante la retirada de dichos péptidos por adsorción en carbón activo, uso de técnicas cromatográficas o la extracción con alcoholes, así como su hidrólisis (Saha & Hayashi, 2001; FitzGerald & O'Cuinn, 2006). No obstante, en ciertos casos no pueden aplicarse estos tratamientos ya que la actividad biológica recae sobre dichos péptidos, o bien los péptidos responsables se ven afectados colateralmente por los tratamientos. Otro factor influyente en las características organolépticas es la necesidad de añadir un ácido o una base para cambiar el pH final de la hidrólisis y así parar la actividad enzimática, repercutiendo en el sabor salino de los hidrolizados finales. La aplicación de métodos de enmascaramiento con otros sabores por adición de otros ingredientes o la técnica de (micro) encapsulación son métodos conocidos para la resolución de problemas organolépticos (Gharsallaoui et al., 2009). Las condiciones del procesamiento industrial y almacenamiento, influidos además por los componentes de la matriz alimentaria, pueden conllevar cambios en la actividad de los péptidos bioactivos a través de diferentes procesos como la racemización, descomposición, glicosilación, entrecruzamiento y oxidación de los aminoácidos (López-Fandiño et al., 2006).

Los países desarrollados han adoptado normativas reguladoras sobre la puesta en el mercado de los alimentos funcionales, como la normativa FOSHU en Japón o aquéllas desarrolladas por la Autoridad Europea de Seguridad Alimentaria y Agencia Estadounidense de Alimentos y Medicamentos. Estudios clínicos en humanos, junto a la demostración de la dosis mínima necesaria para alcanzar el efecto saludable, son requisitos en dichas normativas (Harnedy & FitzGerald, 2012).

La **Tabla 2** presenta una selección de ingredientes comerciales cuya actividad está fundamentada en el efecto de péptidos bioactivos. La introducción en el mercado de dichos ingredientes se ha realizado en un amplio abanico de alimentos, comprendiendo productos lácteos, bebidas de frutas, sopas, confitería, chicles y

productos nutracéuticos, como pastillas y cápsulas (Korhonen, 2009). La mayoría de estos alimentos funcionales se han desarrollado para el tratamiento de la hipertensión moderada, aunque otros efectos fisiológicos como el anticariogénico, hipocolesterolémico, relajante, mejorador del transporte de metales o reductor del índice glucémico, entre otros, también han sido explotados (Choi et al., 2012). No obstante, existen otras funcionalidades biológicas, ya descritas para péptidos bioactivos, que todavía no han sido comercializadas a través de ingredientes relacionados. La fuente proteica más utilizada en la generación de los péptidos bioactivos sigue siendo la leche, aunque se observa una progresiva relevancia de las proteínas de origen marino (Harnedy & FitzGerald, 2012) y vegetal (Howard & Udenigwe, 2013). A los ingredientes comerciales reflejados en la **Tabla 2**, se podrían adjuntar otros que no proceden directamente de una hidrólisis desarrollada sino que surgen de la extracción o enriquecimiento de un péptido generado por procesos alternativos. El caseínmacropéptido o glicomacropéptido (GMP) es un péptido de 64 aminoácidos, derivado de la κ-caseína, que se encuentra en el suero liberado durante la fabricación del queso. El GMP ha demostrado distintas funcionalidades fisiológicas, por ejemplo la anticariogénica mediante la inhibición de la adhesión de la placa bacteriana a las piezas dentales (Aimutis, 2004). Entre otros fabricantes, Davisco ha desarrollado procesos específicos para extraer GMP del suero de quesería y comercializarlo como ingrediente funcional, que se utilizará en distintas matrices. Otro ejemplo similar lo constituye la lunasina, un péptido de 43 aminoácidos presente en el propio grano de soja, y cuya extracción selectiva del aislado de proteína de soja se ha aprovechado para el desarrollo de ingredientes comerciales enriquecidos como Lunasin XP® y LunaSoy™, que ofrecen el efecto hipocolesterolemiante asociado (Udenigwe & Aluko, 2012).

Tabla 2: Selección de ingredientes comerciales basados en el efecto de péptidos bioactivos contenidos en hidrolizados proteicos (Adaptado de Korhonen, 2009; Harnedy & FitzGerald, 2012; Howard & Udenigwe, 2013).

Nombre comercial	Actividad	Fuente	Fabricante
Calpis	Antihipertensivo	Leche fermentada	Calpis Co. (Japón)
Evolus	Antihipertensivo	Leche fermentada	Valio Oy (Finlandia)
LowPept	Antihipertensivo	Hidrolizado de caseínas	Innaves Biotech (España)
Peptide C12	Antihipertensivo	Hidrolizado de caseínas	DMV International (Holanda)
BioZate	Antihipertensivo	Hidrolizado de proteínas de suero	Davisco (EEUU)
Vasotensin	Antihipertensivo	Hidrolizado de bonito	Metagenics (EEUU)
PeptACE	Antihipertensivo	Hidrolizado de bonito	Natural Factors (Canada)
Lapis Support	Antihipertensivo	Hidrolizado de sardina	Tokiwa Yakuhin (Japón)
Valtyron	Antihipertensivo	Hidrolizado de sardina	Senmi Ekisu (Japón)
Soyscience	Hipocolesterolémico	Hidrolizado de soja	Kyowa Hakko Bio (Japón)
Lacprodan DI-2021	Mayor absorción de minerales	Hidrolizado de caseínas	Arla (Dinamarca)
Recaldent	Anticariogénico	Fosfopéptidos de caseínas	Cadbury Enterprises (EEUU)
Nutripeptin	Reducir índice glucémico	Hidrolizado de bacalao	Nutrimarine (Noruega)
Lactium	Relajante	Hidrolizado de caseínas	Ingredia (Francia)
Protizen	Relajante	Hidrolizado de pescado	Copalis (Francia)

2. RESULTS / RESULTADOS

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Food-Derived Peptides Stimulate Mucin Secretion and Gene Expression in Intestinal Cells

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ABSTRACT: In this study, the hypothesis that food-derived opioid peptides besides β-casomorphin 7 might modulate the production of mucin via a direct action on epithelial goblet cells was investigated in HT29-MTX cells used as a model of human colonic epithelium. Seven milk whey or casein peptides, a human milk peptide, and a wheat gluten-derived peptide with proved or probable ability to bind μ- or δ-opioid receptors were tested on the cell culture. Significantly increased secretion of mucins was found after exposure to six of the assayed peptides, besides the previously described β-casomorphin 7, as measured by an enzyme-linked lectin assay (ELLA). Human β-casomorphin 5 and α-lactorphin were selected to study the expression of mucin 5AC gene (MUC5AC), the HT29-MTX major secreted mucin gene. α-Lactorphin showed increased expression of MUC5AC from 4 to 24 h (up to 1.6-fold over basal level expression), although differences were statistically different only after 24 h of exposure. However, this increased expression of MUC5AC did not reach significance after cell treatment with human β-casomorphin 5. In conclusion, six food-derived peptides have been identifed with described or probable opioid activity that induce mucin secretion in HT29-MTX cells. Concretely, α-lactorphin is able to up-regulate the expression of the major secreted mucin gene encoded by these cells.

KEYWORDS: *opioid peptides, milk, gene expression, intestinal goblet cells, mucins*

INTRODUCTION

The gastrointestinal lumen is covered by a viscous solution, known as mucus, which lubricates the epithelia, helping in the passage of substances and particles through the digestive tract, and forms a protective layer against noxious chemicals, microbial infections, dehydration, and changing luminal conditions.[1] The intestinal mucus gel owes its properties to secreted mucins, which are high molecular weight glycoproteins produced by goblet cells of the epithelium. Not surprisingly, mucin gene expression, biosynthesis, and secretion are highly regulated. Disruption of the barrier integrity and invasion of microbes with subsequent chronic inflammation and further disturbance of the mucosal architecture are hallmarks of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.[2] Even colon cancer has been linked to a faulty mucin expression in rat model experiments.[3]

Certain dietary components such as fiber, short-chain fatty acids, and probiotics can positively influence the characteristics of the intestinal mucus, although the mode of action of each compound may differ. Oat bran, rye bran, and soybean hull were shown to increase globlet cell volume density in the proximal and distal small intestine of hamsters.[4] Among the three main short-chain fatty acids produced in the human colon (i.e., acetate, propionate, and butyrate), butyrate appears to be the most effective in stimulating mucus release.[5] The modulation of mucin gene (MUC) expression in intestinal epithelial goblet cells has been subsequently demonstrated.[6] Besides, the mechanisms that regulate butyrate-mediated effects on MUC2 synthesis have been studied.[7] Recently, it has been reported that selected probiotics can induce MUC3 expression of mucosal intestinal epithelial cells in a reproducible, although time-limited, manner.[8]

With regard to dietary proteins, no information about their impact was available until two milk protein hydrolysates (casein and lactalbumin hydrolysates) and the peptide β-casomorphin 7, specifically, were shown to induce a strong release of mucin in the jejunum of the rat through the activation of the enteric nervous system and opioid receptors.[9] Trompette et al.[10] provided evidence that peptides that had shown this effect need to be absorbed and participate in the regulation of intestinal mucus discharge through activation of μ-opioid receptors on intestinal cells. The presence of opioid receptors on these cells suggests the possibility that food-derived peptides with opioid agonist structure, which can be produced in the intestinal lumen during gastrointestinal digestion, might modulate the production of mucin via a direct action on epithelial goblet cells. Rat and human mucus-secreting cell lines can be used as models to avoid interactions with the nervous system. In rat DHE cells, β-casomorphin 7 has been shown to increase mucin secretion and the expression of rat mucin rMuc2 and rMuc3. In human HT29-MTX cells, this peptide increased as well mucin secretion and MUC5AC mRNA levels.[11] The aim of this work was to evaluate if other food peptides with proved or probable

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Table 1. Primers for Real-Time PCR

gene	base pairs	primers		ref
MUC5AC	240	5′-CGACCTGTGCTGTGTACCAT-3′	(2870−2889)	11
		5′-CCACCTCGGTGTAGCTGAA-3′	(3109−3091)	
human cyclophillin	165	5′-TCCTAAAGCATACGGGTCCTGGCAT-3′	(280−304)	11
		5′-CGCTCCATGGCCTCCACAATATTCA-3′	(445−421)	
human β-actin	197	5′-CTTCCTGGGCATGGAGTC-3′	(879−896)	31
		5′-GCAATGATCTTGATCTTCATTGTG-3′	(1076−1053)	

ability to bind μ- or δ-opioid receptors can induce mucin secretion and MUC5AC expression on human HT29-MTX intestinal cells.

MATERIALS AND METHODS

Peptides. Bovine β-casomorphin 7 (YPFPGPI) and (D-Ala2,*N*-Me-Phe4,glycinol5) enkephalin (DAMGO) were purchased from Bachem (Bubendorf, Switzerland). Bovine neocasomorphin (YPVEPF), human β-casomorphin 5 (YPFVE), bovine α-casein exorphin (YLGYLE), bovine β-lactorphin (YLLF-NH_2), human and bovine α-lactorphin (YGLF-NH_2), gluten exorphin A5 (GYYPT), and bovine α-casein fragments 90−94 (RYLGY) and 143−149 (AYFYPEL) were synthesized using conventional solid-phase FMOC synthesis with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK). Their purity (>90%) was verified in our laboratory by reverse phase high-performance liquid chromatography and tandem mass spectrometry.

Cell Culture. HT29-MTX, a human colon adenocarcinoma-derived mucin-secreting goblet cell line, was provided by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France).[12] The cell line was grown in plastic 75 cm^2 culture flasks in DMEM supplemented with 10% FBS and 10 mL/L penicillin−streptomycin solution (all from Gibco, Paisley, UK) at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. Cells were passaged weekly using trypsin/EDTA 0.05% (Gibco). The culture medium was changed every 2 days. To study the effect of peptides or DAMGO, cells were seeded at a density of 5×10^5 cells per well in 12-well culture plates (Nunc, Roskilde, Denmark). The cell line was used between passages 12 and 19. Experiments were performed 21 days after confluency. Twenty-four hours before the studies, the culture medium was replaced by serum- and antibiotic-free medium to starve the cells and to eliminate any interference from extraneous proteins or hormones. After serum-free medium removal, the monolayer was washed twice with PBS. Serum-free medium with or without peptide (0.05−0.5 mM) or DAMGO (0.001 mM) was added to the cells and incubated at 37 °C for 10 min−24 h in a 5% CO_2 humidified atmosphere. The supernatants were collected, frozen, and stored at −70 °C. The total RNA was isolated with Nucleospin RNA II (Macherey-Nagel, Düren, Germany).

Enzyme-Linked Lectin Assay (ELLA). To measure mucin-like glycoprotein secretion, an ELLA reported by Trompette et al.,[13] slightly modified, was used. Briefly, wells of a microtiter plate were coated with sample diluted in sodium carbonate buffer (0.5 M, pH 9.6) and incubated overnight at 4 °C. The plates were then washed with PBS containing 0.1% Tween 20 (PBS−Tween) and blocked with 2% BSA in PBS-Tween (PBS−Tween−BSA) for 1 h at 37 °C. After samples were washed six times, biotinylated wheat germ agglutinin (Vector Laboratories, Peterborough, UK) in PBS−Tween−BSA (1:1000) was added, and the samples were incubated for 1 h at 37 °C. Subsequently, avidin−peroxidase conjugate (Vector Laboratories) (1:50000) was added for signal amplification. Colorimetric determination using *o*-phenylenediamine dihydrochloride solution (Dako, Glostrup, Denmark) was performed at 492 nm.

The mucin-like glycoprotein content of samples was determined from standard curves prepared from gastric porcin mucin (Sigma, Steinheim, Germany). All experiments were performed three times for at least three biological replicates. Data were analyzed by a two-way ANOVA, using GraphPad Prism 4 software, followed by the Bonferroni test for single comparisons. Differences between means and controls were considered to be significant with $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

Real-Time Quantitative RT-PCR Assays (qRT-PCR). Quantitative RT-PCR amplification was carried out with the real-time fluorescence method using a 7500 Fast System (Applied Biosystems). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instruction. The specific primers (target and reference genes) used for the RT-PCR assays are listed in Table 1. The SYBR Green method was used, and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 12.5 μL of 2× SYBR Green real-time PCR Master Mix (Applied Biosystems), 5 μL of 1 μM gene-specific forward and reverse primers, and 2.5 μL of a 1:10 dilution of cDNA. Amplification was initiated at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Control PCRs were included to confirm the absence of primer−dimer formation (no-template control) and to verify that there was no DNA contamination (without RT enzyme negative control). All real-time PCR assays amplified a single product as determined by melting curve analysis and by electrophoresis in 2% agarose gels. A standard curve was plotted with cycle threshold (Ct) values obtained from amplification of known quantities of cDNAs and used to determine the efficiency (E) as $E = 10^{-1/\text{slope}}$.

The relative expression levels of the target gene were calculated using the comparative critical threshold method ($\Delta\Delta$Ct). Human cyclophilin and β-actin were tested as reference genes. The cyclophilin gene was chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used. All experiments were performed at least three times in triplicate ($n = 9$). Data were analyzed by a two-way ANOVA, using GraphPad Prism 4 software. For a better comparison of the concentration versus control data for each time, data were analyzed by a one-way ANOVA, followed by the Newman−Keuls test. Differences between means and controls were considered to be significant with $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$(***).

RESULTS

Determination of Mucin Secretion of HT29-MTX Cell Culture over 24 h. HT29-MTX cells form a homogeneous monolayer of polarized goblet cells that exhibit a discrete apical brush border.[14] Previous studies have shown that the morphological differentiation of the cells, as well as the secretion of mucins when it occurs, is a growth-related phenomenon, starting after the cells have reached confluency.[12] To get quantitative information about the mucin production by HT29-MTX cells, mucins were quantified by ELLA during 24 h, when the proportion of cells that express mucus reaches 100% and remains stable (21 days after confluency).[12] Figure 1 shows the concentration of mucin-like glycoproteins found in the culture medium at increasing times between 30 min and 24 h. The values exhibited a steep increased secretion of mucin between 4 and 8 h (10 times higher) that was followed by a further increase reaching 6 times the 8 h value at 24 h. Close

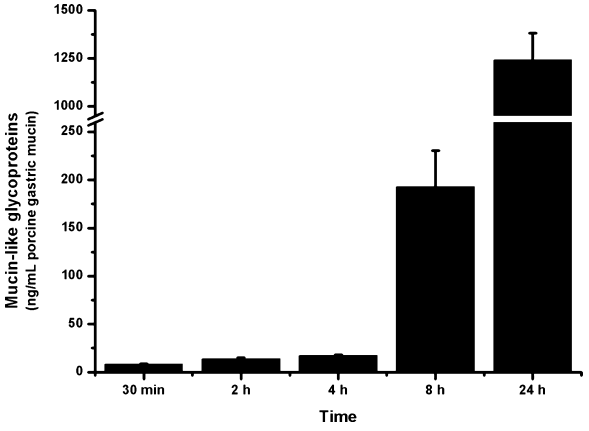

Figure 1. Time course secretion of mucin by HT29-MTX cells determined by enzyme-linked lectin assay. Data are expressed as mucin-like glycoprotein secretion. Each bar represents the mean ± SE of six biological replicates in triplicate.

values were measured in two independent experiments at each time. Therefore, the cell culture proved to be a reliable tool for the study of gastrointestinal mucin secretion.

Mucin Secretion of HT29-MTX Cells under the Effect of Different Food Peptides. Various synthetic milk- or wheat-derived gluten peptides with proved ability to bind μ- or δ-opioid receptors and two casein-derived peptides that had shown a potent antihypertensive effect and sequences that may anticipate opioid activity were added to the cell culture.[15] The specific μ-receptor agonist DAMGO was used as a positive control.

Table 2 summarizes the maximum mucin secretion by HT29-MTX cells after addition of the assayed peptides at 0.1 mM. Six of the eight newly studied food peptides showed significant activity on mucin secretion by HT29-MTX cells. From the casein-derived peptides, human β-casomorphin 5 showed the highest secretion value. Both whey-derived peptides, α-lactorphin and β-lactorphin, showed significantly higher values than the control. Among the studied peptides, gluten exorphin showed the lowest activity with an increase of 157% of control. The specific μ-receptor agonist DAMGO behaved as a potent mucus secretagogue in HT29-MTX cells, as it was used at a 100 times lower concentration than the food-derived peptides. The activity of this agonist had been previously reported in rat ex vivo experiments[10] and DHE cells[11] but not in human cells. From this first screening, the whey-derived peptide, α-lactorphin, and the human β-casomorphin 5 were selected for further experiments.

Figure 2 shows time course experiments of addition of different doses (0.05, 0.1, and 0.5 mM) of α-lactorphin (A) and human β-casomorphin 5 (B) and subsequent determination of secreted mucin by ELLA. Both peptides stimulated the release of mucin-like glycoprotein at 0.5 and 2 h after exposure, which denotes the enhanced mucus discharge in this time range (Figure 2). Although the secretion values did not allow clear evidence of a dose−response effect, in general, higher releases were found with the highest dose (0.5 mM) at 0.5 and 2 h for α-lactorphin and at 0.5 h for human β-casomorphin 5.

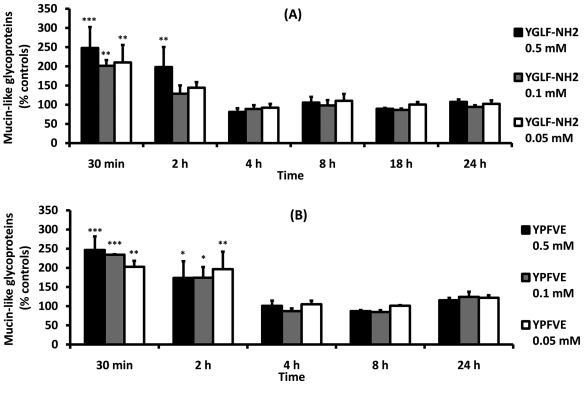

Figure 2. Time course effect at three different concentrations (0.05, 0.1, and 0.5 mM) of α-lactorphin, YGLF-NH2 (A), and human β-casomorphin 5, YPFVE (B), on mucin secretion in HT29-MTX cells determined by enzyme-linked lectin assay. Data are expressed as mucin-like glycoprotein secretion as a percentage of control (untreated cells). Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by two-way ANOVA applying the Bonferroni test: (***)$P < 0.001$; (**) $P < 0.01$; (*) $P < 0.05$.

MUC5AC Expression in HT29-MTX Cells under the Effect of Different Food Peptides. Quantitative PCR was used to determine the level of transcripts of MUC5AC in HT29-MTX cells treated with α-lactorphin and human β-

Table 2. Maximum Mucin Secretion Respect to Control (Untreated HT29-MTX cells) upon Addition of 0.1 mM Different Food Peptides and 0.001 mM DAMGO Determined by Enzyme-Linked Lectin Assay[a]

peptide				
sequence	denomination	food protein	% control	*P*
YPFPGPI	bovine β-casomorphin 7	β-casein A2 f(60−66)	282	<0.001
YPVEPF	bovine neocasomorphin	β-casein f(114−119)	-	
YPFVE	human β-casomorphin 5	β-casein f(51−55)	234	<0.001
YLGYLE	bovine α-casein exorphin 2−7	α-casein f(91−96)	-	
RYLGY		α-casein f(90−94)	191	<0.001
AYFYPEL		α-casein f(143−149)	221	<0.001
YLLF-NH_2	bovine β-lactorphin	β-lactoglobulin f(102−105)	453	<0.001
YGLF-NH_2	bovine and human α-lactorphin	α-lactalbumin f(50−53)	201	<0.001
GYYPT	gluten exorphin A5	wheat glutenin	157	<0.05
	DAMGO		232	<0.01

[a]Data were obtained by three biological replicates in triplicate. Significant differences between average values and control by two-way ANOVA (Bonferroni test).

casomorphin 5. MUC5AC was selected because it is the gene that codifies an abundant secreted mucin and presents high levels of mRNA in HT29-MTX cells.[12] β-Casomorphin 7 was used as positive control, and it showed increased expression levels of MUC5AC after 24 h of exposure (1.7-fold basal level), according to the previously reported results (data not shown).[11] Different concentrations of peptides between 0.05 and 0.5 mM were added to the incubation medium, and cells were incubated during a time range of 10 min−24 h (Figure 3).

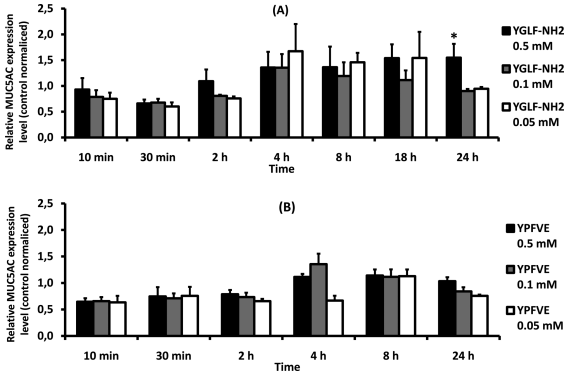

Figure 3. Time course effect at three different concentrations (0.05, 0.1, and 0.5 mM) of α-lactorphin, YGLF-NH2 (A), and human β-casomorphin 5, YPFVE (B), on MUC5AC mRNA level in HT29-MTX cells determined by quantitative RT-PCR. Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophilin as reference gene. Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by one-way ANOVA applying the Newman−Keuls test: (*) $P < 0.05$.

α-Lactorphin showed a trend of increased MUC5AC expression from 4 to 24 h, but, due to the high variability, differences of expression reached significance ($P < 0.05$) only at 24 h (1.6-fold basal level expression at 0.5 mM). On the contrary, time course experiments for human β-casomorphin 5 did not induce a significant increase in MUC5AC mRNA levels compared to untreated cells at the assayed times.

■ DISCUSSION

Mucin secretion by nontreated HT29-MTX goblet cells increased noticeably throughout the 24 h period studied. The slow rise between 0.5 and 4 h may be related to cell adaptation when starvation medium was added. The observed trend is in accordance with information provided in the literature about the high mucin-producing capacity by intestinal goblet cells, based on the important role that mucins play in the epithelium protection and lubrication, as well as its constant renewal.[16]

β-Casomorphin 7 was the first food peptide reported with opioid activity.[17] It is the most studied food-derived opioid peptide, and its influence on the mucin secretion has been evaluated in vitro (human and rat) and ex vivo (rat).[10,11] Its activity on the mucin secretion and MUC5AC expression by goblet cells was confirmed. The present study shows that a whey protein-derived peptide, α-lactorphin, with proved opioid activity, although with lower affinity toward μ-receptors than β-casomorphin 7,[18] can induce mucin secretion and MUC5AC expression. Our results have not found a relationship of the effect of α-lactorphin to dose, because significance in levels of transcripts of MUC5AC was found only at the highest dose assayed (0.5 mM). With regard to the time, α-lactorphin showed increased MUC5AC expression from 4 to 24 h after exposure, although significance was reached at 24 h. The mucin discharge coupled with the corresponding increase of MUC expression to replenish the intracellular mucin pool is a behavior that can be found in other secreting cells such as pancreatic β cells, which respond to changes in blood glucose by first secreting insulin and next increasing insulin biosynthesis.[19] The time range at which mucin exocytosis and stimulation of glycoprotein synthesis reach their maximum under the effect of external agents is still unknown. A study on the treatment of rat cells with butyrate showed that the significant increase in rat mucin gene (rMuc) expression was observed after 24 h but not at earlier time points (1, 3, and 8 h).[6]

Human β-casomorphin 5 showed a significant mucin-secreting activity but no significant overexpression of MUC5AC at the assayed times. Human β-casomorphin 5 displays opioid activity with affinity for μ- and δ-receptors, although it is 2.6 times less potent than β-casomorphin 7.[20]

Two peptides from bovine α-casein, RYLGY and AYFYPEL, showed significant mucin-secretor values. These sequences have not been reported as opioid but have been described in a hydrolysate prepared by our research group for which antihypertensive activity has been demonstrated.[15] Peptide RYLGY is included in the sequence of a casein exorphin (RYLGYL) with moderate opioid activity and μ- and δ-receptor affinity.[21] Peptide AYFYPEL had not been previously described as an opioid peptide but shows an aromatic residue, Tyr, in the second position and Phe together with Tyr in the third and fourth positions, respectively, which forms a favorable structure to bind opioid receptors.[22] Gluten exorphin A5, a peptide having demonstrated opioid activity[23] presented mucin secretion activity. The low value encountered is in accordance with the lower μ-receptor affinity of this peptide compared to δ-receptor.[23] Even so, this is the first report of the mucin-secretory activity of this opioid peptide of vegetal origin on human HT29-MTX goblet cells.

Finally, peptides showing no stimulatory activity, neocasomorphin and α-casein exorphin 2−7, although having been previously reported as opioid peptides, have shown lower activity affinity for μ- and δ-receptor than β-casomorphin 7.[24] However, this lower affinity cannot explain the lack of activity found for these peptides, because the affinity of neocasomorphin for μ-receptors is higher than that of α-lactorphin. Although it has been described that enzymatic activity and expression of intestinal peptidases are lower in HT29 compared with Caco-2 cells,[25] it is possible that some of these sequences are susceptible to the action of cell peptidases and, therefore, peptides could be hydrolyzed to an inactive form. This point will be considered in future studies because it can explain the lack of activity of previously described opioid sequences.

The fact that not only casein-derived but also whey-derived peptides provoke stimulation of mucin secretion and modulation of mucin expression in goblet cells opens a new perspective, with regard to previous works, wherein β-casomorphins were solely considered to play an important physiological role in this cell type. In fact, casein has demonstrated in vivo an effect on intestinal mucin expression in the rat, whereby a significant increase of rMuc3 mRNA in the small intestinal tissue and rMuc4 mRNA in the colon has been observed, when a diet containing hydrolyzed casein compared

to a synthetic amino acid mixture or a protein-free diet was orally administered.[26] There have been studies related to the administration of bovine α-lactalbumin and the stimulation of mucus metabolism in gastric mucosa,[27,28] and some reports had evidenced the activity of α-lactalbumin and hydrolysates of this protein on gastric ulcer on rat models in vivo.[29] Furthermore, a hydrolysate of this protein induced a strong release of mucin in the jejunum of the rat ex vivo.[9] However, some researchers support the view that the protection by whey protein on induced colitis in rats has to be attributed to its high content in threonine and cysteine and to a reduced gene expression of inflammation markers such as interleukin 1β, calprotectin, and inducible nitric oxide synthase.[30] Indubitably, the mechanisms involved in the protective effect of dietary peptides on gastrointestinal mucosa need to be ascertained.

In conclusion, six food-derived peptides have been shown to induce mucin secretion in HT29-MTX human colonic goblet-like cells for the first time. Some of them had been previously described as opioid peptides but two sequences had not, although their structure may be favorable to bind opioid receptors. Concretely, α-lactorphin increased the expression of MUC5AC. This is a first step in finding new food peptides that can be included in the wide variety of stimuli that provoke mucin secretion in goblet cells and therefore play a role in the modulation of this protective function. These findings may assist in the development of dietary strategies to augment mucus layer formation as protection against inflammatory bowel disease effects.

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Notes

The authors declare no competing financial interest.

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Effect of β-lactoglobulin hydrolysate and β-lactorphin on intestinal mucin secretion and gene expression in human goblet cells

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ABSTRACT

A hydrolysate obtained from a whey protein concentrate rich in β-lactoglobulin has been shown to stimulate mucin secretion and mucin 5AC gene expression in human intestinal goblet cells HT29-MTX. Mass spectrometry-based peptidomic analysis allowed the identification of the peptides contained in the hydrolysate. None of them had the required structure to bind opioid receptors, except for the sequence YLLF, corresponding to β-lactorphin. The exposure of the cells to synthetic β-lactorphin evoked a mucin secretory effect although no change in the mucin gene expression was observed. The amidated homolog of this peptide, which has been reported as a more potent opioid, induced mucin gene expression after incubation for 4 h. This supports the hypothesis that induction of the mucin secretory response can be mediated by μ-opioid receptors in HT29-MTX cells, although other mechanisms responsible for the hydrolysate activity cannot be excluded. Protein hydrolysates with the ability to induce mucin secretion could be promising for improving gastrointestinal protection.

1. Introduction

Specialized cells, named goblet cells, are distributed throughout the intestinal epithelium and are responsible for synthesis and secretion of mucins, the major components of the mucus gel (Montagne, Piel, & Lalles, 2004). In healthy animals, the epithelium mucus gel is in a dynamic balance between its release by goblet cells and its loss based on proteolytic and physical processes. One interesting feature of intestinal epithelial cells is that they are always exposed to high concentrations of nutrients, non-nutrients, microbes, and xenobiotic chemicals. This means that, although functions of intestinal epithelial cells are generally controlled by internal factors such as hormones and cytokines, they are also influenced or even regulated by external substances, including food components (Shimizu, 2010). Dietary regulation of intestinal mucin dynamics has potential to contribute to the prevention and management of inflammatory bowel disease effects. Some findings raised by different groups have shown the positive role that food components such as short chain fatty acids (Burger van Paassen et al., 2009; Gaudier et al., 2004), probiotics (Dykstra et al., 2011) or peptides can play. Regarding the last, casein and α-lactalbumin hydrolysates and, specifically, the opioid peptide β-casomorphin 7, have been shown to induce an increased mucin release in rat jejunum. This effect was apparently triggered by a neural pathway and mediated by μ-opioid receptors (Claustre et al., 2002; Trompette et al., 2003). The direct action of β-casomorphin 7 was also demonstrated in rat DHE cells and human HT29-MTX cells, via the activation of μ-opioid receptors which are localized on the membrane of these intestinal goblet cells. In addition to induced mucin secretion, an increase in transcripts for rat mucin genes (rMuc) 2 and 3 and human mucin 5AC gene (MUC5AC) was revealed (Zoghbi et al., 2006). Moreover, in the rat, hydrolyzed casein has been shown to up-regulate in vivo rMuc3 and rMuc4 expression (Han, Deglaire, Sengupta, & Moughan, 2008).

In a previous screening with different food peptides with proved or probable ability to bind opioid receptors, it was observed that some of them induced mucin secretion in HT29-MTX cells (Martínez-Maqueda et al., 2012). Among the peptides studied, it was found that an α-lactalbumin-derived peptide, α-lactorphin, stimulated mucin secretion and induced an increase of MUC5AC expression. The amidated form of β-lactorphin (YLLF-NH_2), which had previously been described as an opioid peptide (Yoshikawa, Fumito, Takashi, & Hideo, 1986), showed significant activity on mucin secretion in HT29-MTX cells, although the effect of this peptide on gene expression was not studied. The aim of this study was to determine if a whey protein hydrolysate containing β-lactorphin, and this peptide specifically, could modulate mucin secretion and expression in human goblet cells HT29-MTX.

2. Materials and methods

2.1. Samples

A bovine whey protein concentrate (WPC) rich in β-lactoglobulin was purchased from Friesland Foods Domo (Zwolle, The Netherlands). It contained a minimum protein content of 84% on dry matter from

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which at least 99.0% corresponded to β-lactoglobulin, as determined by SDS-PAGE and matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) analysis. The whey protein concentrate was dissolved in water 5% (w/v) and heated at 90 °C for 10 min at pilot scale. Hydrolysis was carried out in triplicate by food-grade porcine trypsin (Biocatalysts, Nantgarw, Wales, UK) at 37 °C and pH 8.0 by addition of 1 M NaOH (food grade, Aditio, Panreac Química, S.A.U., Castellar del Vallès, Spain) for 3 h with constant agitation. Porcine trypsin was added at the enzyme-to-substrate ratio of 1:20 (w/w). Reactions were stopped by heating at 95 °C for 15 min, assuring the complete inactivation of the enzyme. The hydrolysate was dried by spray drying. The hydrolysate was analyzed for total amino acid content with a Biochrom 20 plus amino acid analyzer (Amersham-Pharmacia Biotech).

Bovine β-lactorphin (YLLF) was purchased from Genscript (Piscataway, NJ, USA). Amidated β-lactorphin (YLLF-NH_2) was synthesized using conventional solid-phase FMOC synthesis with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK). Their purity (>95%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry (RP-HPLC-MS/MS).

2.2. Cell culture

HT29-MTX cell line, a human colon adenocarcinoma-derived mucin-secreting goblet cell line (Lesuffleur et al., 1993), was grown as described previously (Martínez-Maqueda et al., 2012). Experiments were conducted between passages 17 and 24. Serum-free medium with (0.05, 0.1, and 0.5 mM) or without peptide or WPC hydrolysate (0.1, and 1% w/v) was added to cells and incubated at 37 °C for 2 to 24 h. The supernatants were collected, frozen and stored at −70 °C. The total RNA was isolated with Nucleospin® RNA II (Macherey-Nagel, Düren, Germany).

2.3. Enzyme-linked lectin assay

Mucin-like glycoprotein secretion was determined by an enzyme-linked lectin assay (ELLA), as previously reported (Martínez-Maqueda et al., 2012). All experiments were performed three times for at least three biological replicates.

2.4. Quantitative RT-PCR assays (qRT-PCR)

Quantitative RT-PCR amplification was carried out using a Lightcycler 480 (Roche, Mannheim, Germany) in 384 wells microplates (Roche). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instruction. For MUC5AC (accession no. AJ001402), target gene, primers 2870-2889/3109-3091 were used. For reference genes cyclophillin (accession no. Y00052) and β-actin (accession no. NM_001101) primers 280-340/445-421 and 879-896/1076-1053, respectively, were used (Tai et al., 2008; Zoghbi et al., 2006). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 5 μL 2× SYBR Green real-time PCR Master Mix (Roche) 0.25 μL of a 10 μM of gene-specific forward and reverse primers, 0.27 μL of cDNA and 4.23 μL of water. Amplification was initiated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Control PCRs were included to confirm the absence of primer dimer formation (no-template control), and to verify that there was no DNA contamination (without RT enzyme negative control). All real-time PCR assays amplified a single product as determined by melting curve analysis.

The relative expression levels of the target gene were calculated using the comparative critical threshold method (ΔΔCt). Human cyclophilin and β-actin were tested as reference genes. Cyclophilin gene was chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used. All experiments were performed at last three times in triplicate.

2.5. Analysis by HPLC-tandem mass spectrometry

RP-HPLC-MS/MS characterization of the hydrolysate and YLLF quantification were performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionization source as previously described (Contreras et al., 2010). The column used was a reverse phase XBridge PST C18 Column (150×2.1 mm i.d., 5 μm particle size) (Waters Corp, Milford, MA, USA). The hydrolysate was dissolved at the concentration of 5 mg/mL. The signal threshold to perform tandem mass spectrometry was 50,000. To aid the identification of disulfide-linked fragments, the hydrolysate was also analyzed after a reducing step using dithiothreitol (DTT), at a final concentration of 70 mM and pH 7, for 1 h at 37 °C, as described previously (Chicón, López-Fandiño, Alonso, & Belloque, 2008). The quantification was performed by representing the peak obtained by MS analysis vs. the YLLF concentration. Plots were made with the peak area of the molecular ions with m/z value of 555.3, corresponding to YLLF, and their sodium and potassium adducts (m/z 577.3 y 593.3, respectively). Six calibration points were obtained from 1 to 32 μg/mL.

To study peptide stability during experiments, cell supernatants were also analyzed by RP-HPLC-MS/MS using a reverse phase Mediterranea™ Sea C18 Column (150×2.1 mm i.d., 5 μm particle size) (Teknokroma, Barcelona, Spain). The samples were eluted at 0.2 mL/min and the eluent from the HPLC was entirely directed to the mass spectrometer.

Data obtained were processed and transformed to spectra representing mass values using the Data Analysis program (version 4.0, BrukerDaltonik). To process the MS/MS spectra and to perform peptide sequencing BioTools (version 3.1, BrukerDaltonik) was used.

2.6. Statistical analysis

Data were analyzed by a two-way ANOVA, followed by the Bonferroni test. For a better comparison of the concentrations vs control data for each time, data were analyzed by a one-way ANOVA, followed by the Newman–Keuls test. GraphPad Prism 4 software was used to find significant differences between means and controls as $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$(***).

3. Results

3.1. Characterization of WPC hydrolysate

In order to evaluate the activity of a hydrolysate containing β-lactorphin on HT29-MTX cells, a tryptic hydrolysate of a β-lactoglobulin enriched WPC was prepared. β-lactorphin had been previously obtained by hydrolysis with pepsin and trypsin (Antila et al., 1991). Preliminary studies at our laboratory showed that the use of food grade trypsin also allowed the release of this peptide. Peptidomic analysis was performed under different conditions at the mass spectrometer to cover short and longer peptides, and with and without DTT reduction to detect disulphide linked peptides. Table 1 shows the identified peptides in the WPC hydrolysate. A total of 45 β-lactoglobulin peptide sequences were identified being the protein coverage 97.5%. In addition to individual peptides, 4 heterodimers corresponding to peptides linked by a disulphide bond could be identified by comparing the reduced and non reduced sample. An additional Cys-containing peptide f(110–124) was also identified in the reduced sample but the Cys-containing fragment that could be linked to the first was not sequenced by tandem mass spectrometry, although the experimental mass corresponding to the heterodimer (Tyr_{102}–Asn_{109}) S-S(Ser_{110}–Arg_{124}) was observed in the non-reduced sample. Among

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Table 1
Peptides identified in the tryptic hydrolysate of the whey protein concentrate enriched in β-lactoglobulin. Disulphide linked peptides were only detected in the non reduced sample and are marked with an asterisk.

Experimental mass	Theoretical mass	β-Lactoglobulin fragment	Sequence
932.6	932.536	1–08	LIVTQTMK
819.5	819.452	2–08	IVTQTMK
672.6	672.381	8–14	GLDIQK
387.4	387.248	12–14/58–60	IQK/LQK
695.5	695.328	15–20	VAGTWY
1191.1	1190.622	21–32	SLAMAASDISLL
788.5	788.428	25–32	AASDISLL
646.6	646.354	27–32	SDISLL
856.6	856.440	33–40	DAQSAPLR
542.5	542.318	36–40	SAPLR
1943.0	1943.014	41–57	VYVEELKPTPEGDLEIL
1680.9	1680.882	43–57	VEELKPTPEGDLEIL
2050.0	2050.120	43–60	VEELKPTPEGDLEILLQK
658.6	658.354	52–57	GDLEIL
1121.5	1120.437	61–69	WENDECAQK
1249.4	1248.532	61–70	WENDECAQKK
1876.4	1875.736	(61–69) S-S(157–162)*	(WENDECAQK)S-S(EEQCHI)
1158.3	1157.989	(61–69) S-S(153–162)*	(WENDECAQK) S-S(PTQLEEQCHI)
1387.7	1388.603	(61–69) S-S(149–162)*	(WENDECAQK) S-S(LSFNPTQLEEQCHI)
1397.0	1396.108	(61–70) S-S(150–162)*	(WENDECAQKK) S-S(SFNPTQLEEQCHI)
700.7	700.448	70–75	KIIAEK
572.5	572.353	71–75	IIAEK
774.6	774.464	76–82	TKIPAVF
902.5	902.559	76–83	TKIPAVFK
545.4	545.321	78–82	IPAVF
673.7	673.416	78–83	IPAVFK
1043.6	1043.561	83–91	KIDALNENK
915.6	915.466	84–91	IDALNENK
1064.6	1064.583	92–100	VLVLDTDYK
1192.7	1192.670	92–101	VLVLDTDYKK
853.4	852.423	94–100	VLDTDYK
554.4	554.310	102–105	YLLF
1660.7	1658.776	110–124	SAEPEQSLVCQCLVR
1244.1	1244.577	125–135	TPEVDDEALEK
1634.2	1634.768	125–138	TPEVDDEALEKFDK
1436.6	1434.667	127–138	EVDDEALEKFDK
1046.4	1046.477	127–135	EVDDEALEK
836.6	836.469	142–148	ALPMHIR
424.4	424.255	146–148	HIR
1304.6	1304.625	149–159	LSFNPTQLEEQ
918.5	918.481	149–156	LSFNPTQL
1657.8	1656.769	149–162	LSFNPTQLEEQCHI
1544.6	1543.685	150–162	SFNPTQLEEQCHI
1196.4	1195.542	153–162	PTQLEEQCHI
757.4	756.299	157–162	EEQCHI

the fragments bound with a disulphide bond, peptide (Trp_{61}–Lys_{69}) S-S(Glu_{157}–Ile_{162}) was the most abundant. Most fragments corresponded to the specific trypsin cleavages at arginine and lysine sites although some of them ended by hydrophobic residues indicating that the food grade trypsin used also contains other enzymatic activities. Some of the sequences found in the hydrolysate had been previously described as biologically active peptides. This is the case of the hypocholesterolemic peptides IIAEK and GLDIQK (Nagaoka et al., 2001). Among the identified peptides, only the sequence YLLF, corresponding to β-lactorphin, had the required structure to bind opioid receptors, i.e., tyrosine at the amino-terminal end and another aromatic residue at the third or fourth position and had previously been described as opioid peptide (Antila et al., 1991). The amount of this peptide was quantified by an external calibration curve with the synthetic peptide and it was around 0.68 mg/g (calibration curve $y = 1.4\times10^6 - 2.2\times10^6$, $R^2 = 0.996$).

3.2. Effect of WPC hydrolysate on mucin secretion and expression of HT29-MTX cells

To investigate a possible effect of the hydrolysate on mucin secretion and MUC5AC expression, HT29-MTX cells were treated with two different concentrations of the product (0.1 and 1% w/v) during 2, 4, 8 and 24 h. The secretion of mucin by cells was monitored by determining the mucin-like glycoproteins in the cell supernatants. A significant ($P<0.05$) increase of mucin secretion (152% of control) was observed at 2 h of exposure to 0.1% of hydrolysate. The concentration of mucin-like glycoproteins in the supernatant, after 8 h of incubation, was also higher than that from the control cells, although the difference did not reach significance (Fig. 1A). It was not possible to perform the mucin determination in the supernatants containing 1% of hydrolysate probably due to the interference of lactose or peptides at high concentration with the ELLA reagents. To quantify the effect of the hydrolysate on the major secreted mucin gene encoded by HT29-MTX cells, MUC5AC, its transcription level was determined by qRT-PCR. As shown on Fig. 1B, the relative expression of the gene under the influence of the hydrolysate significantly raised after 4 h. At the higher concentration (1%), the values reached 1.53-fold basal level ($P<0.05$). At 24 h, no substantial differences were observed compared to controls.

3.3. Effect of β-lactorphin on mucin secretion and expression of HT29-MTX cells

To assess the direct effect of β-lactorphin on HT29-MTX cells, three different concentrations (0.05, 0.1 and 0.5 mM) of this peptide were

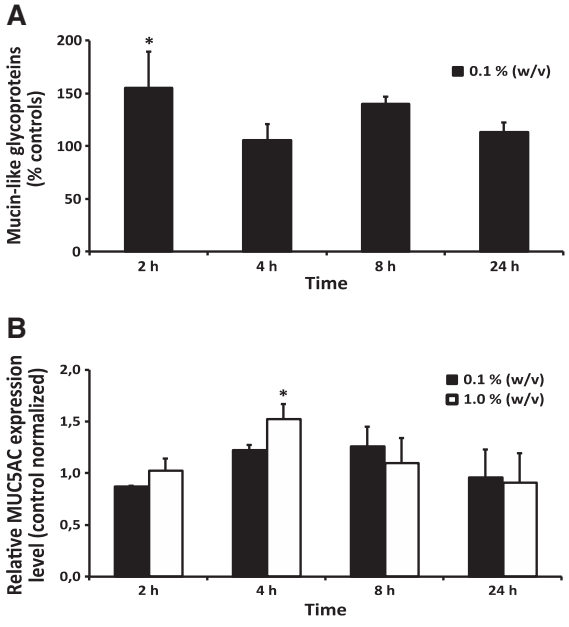

Fig. 1. Time-course effect of the whey protein concentrate hydrolysate in HT29-MTX cells. A) mucin secretion determined by enzyme-linked lectin assay (ELLA) after addition of the whey protein hydrolysate at the concentration of 0.1% (w/v). Data are expressed as mucin-like glycoprotein secretion as a percentage of control (untreated cells). Significant differences of each concentration vs control were determined by two-way ANOVA applying the Bonferroni test. B) MUC5AC mRNA level determined by quantitative RT-PCR (qRT-PCR) after addition of the hydrolysate at two different concentrations (0.1 and 1%). Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophillin as reference gene. Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration vs control were determined by one-way ANOVA applying the Newman–Keuls test:*$P<0.05$.

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added to the cell culture and it was incubated during 2, 4, 8 and 24 h. Fig. 2A shows the mucin secretion after the exposure of the cells to β-lactorphin. The maximum increase (174% of control, $P<0.05$) was observed after 2 h at the highest peptide concentration (0.5 mM). In order to know if the synthetic peptide presented an effect on the expression of MUC5AC, qRT-PCR was used. The highest MUC5AC expression level was found after 2 h of exposure (1.26-fold basal level) but no significant differences were observed between treated and control cells (Fig. 2B). An estimation of the stability of the peptide in the supernatants was performed. The area of β-lactorphin peak was monitored along the time of exposure until 24 h. Up to 4 h, 85% of the initial area was kept but a sharp decrease was observed at 24 h (25% of the initial area). Shorter forms from the peptide, which could reveal degradation of the peptide due to the action of cell peptidases, were not detected in the supernatants. Therefore, the decrease of the peptide concentration with the incubation time could be attributed to the absence of peptide in the withdrawn supernatant due to its localization in the mucin layer or intracellularly.

β-lactorphin belongs to the opioid-like structure family of peptides Tyr-X_1-X_2-Phe, and it had been reported that the amide form of this peptide exerts opioid activity (Yoshikawa et al., 1986). In a previous study, we have found that the amidated β-lactorphin elicited a high mucin secretion (453% of control) upon exposure to HT29-MTX cells at a concentration 0.1 mM (Martínez-Maqueda et al., 2012). Therefore, its activity on MUC5AC expression was also studied in HT29-MTX cells at three different concentrations (0.05, 0.1 and 0.5 mM) (Fig. 3). The amidated β-lactorphin significantly increased ($P<0.05$) the relative basal MUC5AC mRNA level up to 2.22 fold when HT29-MTX cells were treated with a 0.5 mM concentration during 4 h. The values in the time-range from 4 to 24 h remained increased with respect to untreated cells.

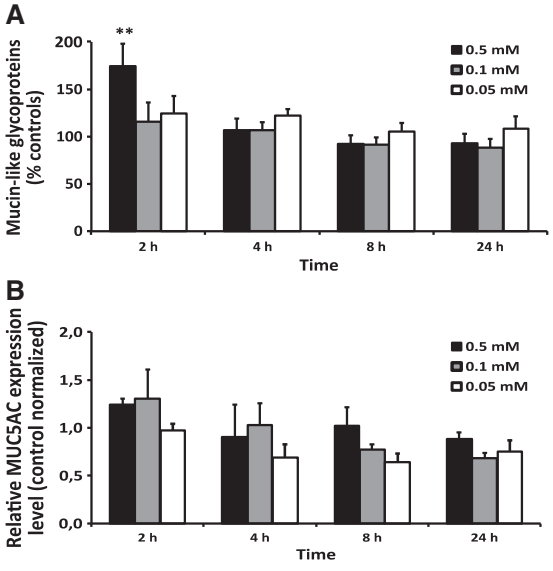

Fig. 2. Time-course effect at three different concentrations (0.05, 0.1 and 0.5 mM) of synthetic β-lactorphin (YLLF) in HT29-MTX cells. A) Mucin secretion determined by enzyme-linked lectin assay (ELLA). Data are expressed as mucin-like glycoprotein secretion as a percentage of control (untreated cells). B) MUC5AC mRNA level in HT29-MTX cells determined by quantitative RT-PCR (qRT-PCR). Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophillin as reference gene. Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration vs control were determined by two-way ANOVA applying the Bonferroni test: ** $P<0.01$.

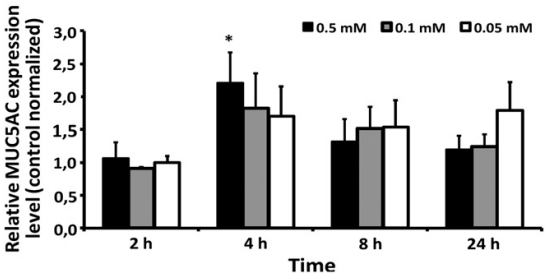

Fig. 3. Time-course effect at three different concentrations (0.05, 0.1 and 0.5 mM) of synthetic amidated β-lactorphin (YLLF-NH_2) on MUC5AC mRNA level in HT29-MTX cells determined by quantitative RT-PCR (qRT-PCR). Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophillin as reference gene. Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration vs control were determined by two-way ANOVA applying the Bonferroni test: * $P<0.05$.

4. Discussion

The present study shows the activity of a β-lactoglobulin hydrolysate, containing β-lactorphin, on mucin secretion and gene expression in intestinal human goblet cells HT29-MTX. Maximum secretion preceded gene expression stimulation. This is in accordance with the statement that mucin-regulated discharge is coupled with the corresponding increase of MUC gene expression, which may just reflect the need to replenish the intracellular mucin pool of goblet/mucous cells (Perez-Vilar, 2009).

The peptide identified in the hydrolysate that most likely could induce mucin secretion due to its ability to interact with opioid receptors was the fragment 102–105 of β-lactoglobulin, β-lactorphin (Antila et al., 1991). In fact, synthetic β-lactorphin increased mucin secretion at the highest concentration (0.5 mM) but did not change to a significant degree the relative expression of MUC5AC. The concentration of β-lactorphin assayed, when using the hydrolysate at 1%, was lower than the concentration used in the pure peptide assay. It is conceivable that, in addition to β-lactorphin, other peptides could contribute to the mucin stimulatory effect. On the other hand, the fact that the amidated form, YLLF-NH_2, which has a more potent opioid activity than the non-amidated peptide (Yoshikawa et al., 1986), had a clear effect on MUC5AC expression reinforces the μ-opioid mediated mechanism proposed for this activity.

Frequently, the mechanisms that explain peptide activities involve more than one pathway. It has been reported that cheese whey protein increases fecal mucin secretion in rats exposed to dextran sulfate sodium (DSS)-induced inflammation of the large intestine without affecting expression of rMuc2 (Sprong, Schonewille, & van der Meer, 2010). The protective effect was linked to the content of threonine and cysteine as amino acids with limiting availability for mucin synthesis under conditions of intestinal inflammation. Previously, it had been shown that supplementation with cysteine, threonine, proline and serine increases mucin synthesis and improves histological changes in DSS-induced colitis in rats, again without changes in mRNA levels of rMuc2 (Faure et al., 2006). The amino acid analysis revealed a negligible amount of free amino acids in the hydrolysate (insignificant amount of free cysteine was found) and the composition of the total amino acid content was similar to that of β-lactoglobulin (data not shown). Certainly, the presence in the hydrolysate of the cited amino acids might affect mucin production at the translational level, but the role of whey peptides at the transcriptional level has also to be taken into consideration.

In conclusion, a WPC hydrolysate, containing β-lactorphin, is able to stimulate mucin secretion and MUC5AC expression in human intestinal goblet cells HT29-MTX. Therefore, whey protein hydrolysates with the ability to modulate mucin production could be promising for improving gastrointestinal protection. This activity could be mediated

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through an opioid pathway although alternative mechanisms cannot be excluded. To confirm this aspect, further experiments with opioid antagonists or β-lactoglobulin hydrolysates, excluding β-lactorphin, are necessary. Studies of administration of the hydrolysate in an animal model are already in progress.

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Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells

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ABSTRACT

The present study was undertaken to explore if a casein hydrolysate and four component peptides with probable ability to interact with opioid receptors can exert a stimulatory effect on mucin production in human intestinal cells (HT29-MTX). α_{S1}-Casein fragments 143–149 (AYFYPEL) and 144–149 (YFYPEL), and the casein hydrolysate, significantly increased expression of MUC5AC, the major secreted mucin gene in this cell line, over 1.7-fold basal level after 4 h of exposure. The determination of mucin-like glycoproteins showed a higher effect on mucin secretion by the casein hydrolysate (210% of controls) than that of AYFYPEL and YFYPEL (around 160%). Therefore, peptides or other components may participate in the activity of the hydrolysate in a synergistic way or through a non-opioid mechanism. In conclusion, a casein hydrolysate and two derived peptides, AYFYPEL and YFYPEL, promote the mucin production and may support the development of functional foods to improve mucus barrier and its protective role in gastrointestinal diseases.

1. Introduction

The role of proteins, either directly or after hydrolysis, as physiologically active components in the diet is being increasingly acknowledged. Peptide sequences can be released from the precursor food proteins by in vivo gastrointestinal digestion and by in vitro processes such as fermentation, controlled enzymatic hydrolysis or reactions occurring during food storage (e.g., cheese ripening). The term "food-derived bioactive peptides" is related to dietary peptide sequences that may exert an in vivo biological function. Among them, interest is growing in those peptides that exhibit effects on the intestine, especially modulatory activities (Shimizu, 2010). There are several reports describing the role of food-derived bioactive peptides increasing gut secretory and absorptive capacity (Moughan, Fuller, Han, Kies, & Miner-Williams, 2007). For instance, the release of hormones such as cholecystokinin, gastrin, and somatostatin can be influenced by peptide hydrolysates (Foltz et al., 2008). The intestinal epithelium constitutes a large surface between the body and the exterior milieu, being continuously exposed to food toxins, pathogens and the changing luminal conditions such as pH changes or the action of proteolytic enzymes. These factors may have a negative impact on the functions of intestinal cells.

The intestinal mucus layer plays a protective role as a barrier between the epithelium and the luminal content. Mucins, which are high molecular weight glycoproteins, represent the main component responsible for the intestinal mucus structure and its protective properties. Mucins are produced by goblet cells and any quantitative or qualitative modification of their synthesis may affect the efficiency of the protection. Fortunately, certain dietary components have been shown to positively influence the producing of mucus. Examples of these dietary components are some short-chain fatty acids and dietary fibres that increase mucin synthesis or the goblet cell number (Barcelo et al., 2000; Gaudier et al., 2004). Of note, a casein-derived peptide with reported opioid activity, β-casomorphin 7, exhibited an enhanced mucin secretion and mucin gene over-expression mediated by μ-opioid receptors in two models (human and rat) of intestinal goblet cells (Zoghbi et al., 2006). In rat jejunum, it has been also demonstrated ex-vivo that luminal administration of β-casomorphin 7 and commercial hydrolysates of casein and α-lactalbumin induced mucin release through a nervous pathway and opioid receptor activation (Claustre et al., 2002; Trompette et al., 2003). In a screening with different food peptides, it was found that several peptides produced a significant increase in secretion of mucins. Furthermore, α-lactorphin showed enhanced expression of the mucin gene 5AC (MUC5AC) in intestinal cells (Martínez-Maqueda et al., 2012). Recently, the total peptide pool from a fermented milk showed its effect on the stimulation of gel-forming MUC2 expression as well as mucin secretion in HT29-MTX cells (Plaisancié et al., 2013).

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Dietary opioid peptides have not been shown to reach the blood stream system in adult animals or humans (Read, Lord, Brantl, & Koch, 1990), although the physiological activities have been unequivocally associated. Therefore, there is a growing line of evidence indicating that at least β-casomorphins (β-casein-derived opioid peptides) may have a local regulatory role on the gastrointestinal tract in adults (Teschemacher, 2003). Milk opioid peptides are generated by hydrolysis of caseins and whey proteins (Teschemacher, Koch, & Brantl, 1997), that occurs both in enzymatic hydrolysates and in vivo gastro-intestinal digestion of dairy products (Svedberg, De Haas, & Leimenstoll, 1985). Furthermore, processing of commercial products can promote the release of opioid peptides, for instance, in cheeses (De Noni & Cattaneo, 2010; Sienkiewicz-Szłapka et al., 2009), and infant formulae (Jarmołowska et al., 2007). β-Casomorphin 7 has also been identified in simulated gastrointestinal digestion of an infant formula (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004).

Interestingly, in a peptic casein hydrolysate with antihypertensive properties, the peptides with major activity were identified as α_{S1}-casein fragments: 90–94 (RYLGY), which differ in one amino acid at the C-terminal end from a previously described opioid peptide (RYLGYL) (Loukas, Varoucha, & Zioudrou, 1983), and 143–149 (AYFYPEL), which has a favourable structure to bind opioid receptors due to the presence of Tyr in the second position and Phe together with Tyr in the third and fourth positions, respectively (Meisel & Fitzgerald, 2000). In a recent screening, both fragments showed significant activity on mucin secretion in human intestinal cells (Martínez-Maqueda et al., 2012). Moreover, other peptides identified in this hydrolysate, although not previously reported as opioid peptides, exhibit amino acid sequences that could be predicted to interact with opioid receptors, such as α_{S1}-casein f(144–149) (YFYPEL) and α_{S2}-casein f(89–95) (YQKFPQY). The present study was undertaken to explore if this casein hydrolysate and derived peptides with probable ability to interact with opioid receptors can exert a stimulatory effect on mucin secretion and gene expression in human intestinal cells.

2. Materials and methods

2.1. Samples

The bovine α_{S1}-casein fragments 90–94 (RYLGY), 143-149 (AYFYPEL), and 144–149 (YFYPEL) were synthesised using conventional solid-phase FMOC synthesis with a 433A peptide synthesiser (Applied Biosystems, Warrington, UK). Their purity (>90%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry. Bovine α_{S2}-casein fragment 89–95 (YQKFPQY) was synthesised by Genscript Corporation (Piscataway, NY, USA). The casein hydrolysate (Lowpept®) was prepared by casein hydrolysis with food-grade pepsin (Biocatalysts, Cardiff, UK) as previously described (Contreras, Carrón, Montero, Ramos, & Recio, 2009). The resulting hydrolysed casein was subsequently spray-dried to produce a dried powder.

2.2. Cell culture

HT29-MTX cell line, a human colon adenocarcinoma-derived mucin-secreting goblet cell line (Lesuffleur et al., 1993), was grown as described previously (Martínez-Maqueda et al., 2012). Experiments were conducted between passages 17 and 23. Serum-free medium with or without peptide (0.05, 0.1, and 0.5 mM) or casein hydrolysate (0.1 and 1%, w/v) was added to cells and incubated for 2–24 h at 37 °C. The supernatants were collected, frozen and stored at −70 °C. The total RNA was isolated with Nucleospin® RNA II (Macherey-Nagel, Düren, Germany).

2.3. Enzyme-linked lectin assay

Mucin-like glycoprotein secretion was determined by an enzyme-linked lectin assay (ELLA), as previously reported (Martínez-Maqueda et al., 2012). All experiments were performed three times with at least three biological replicates.

2.4. Real-time quantitative RT-PCR assays (qRT-PCR)

Quantitative RT-PCR amplification was carried out using a Lightcycler 480 (Roche, Mannheim, Germany) in 384-well microplates (Roche). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For MUC5AC (accession no. AJ001402), target gene primers 2870-2889/3109-3091 were used. For reference genes cyclophillin (accession no. Y00052) and β-actin (accession no. NM_001101) primers 280-340/445-421 and 879-896/1076-1053, respectively, were used (Tai et al., 2008; Zoghbi et al., 2006). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 5 μL 2× SYBR Green real-time PCR Master Mix (Roche) 0.25 μL of a 10 μM of gene-specific forward and reverse primers, 0.27 μL of cDNA and 4.23 μL of water. Amplification was initiated at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Control PCRs were included to confirm the absence of primer dimer formation (no-template control), and to verify that there was no DNA contamination (without RT enzyme negative control). All real-time PCR assays amplified a single product as determined by melting curve analysis.

The relative expression levels of the target gene were calculated using the comparative critical threshold method (ΔΔCt). Human cyclophilin and β-actin were tested as reference genes. Cyclophilin gene was chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used. All experiments were performed at last three times in triplicate.

2.5. Analysis by reverse phase high-performance liquid chromatography-tandem mass spectrometry

Reverse phase high-performance liquid chromatography-tandem mass spectrometry (RP-HPLC-MS/MS) quantification of peptides AYFYPEL, YFYPEL, RYLGY, and YQKFPQY in the hydrolysate was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionisation source as previously described (Contreras et al., 2010). The column used was a reverse phase XBridge PST C18 Column (150 × 2.1 mm i.d., 5 μm particle size) (Waters Corp, Milford, MA, USA). The signal threshold to perform tandem mass spectrometry was 50,000. The hydrolysate was dissolved at the concentration of 2.5 mg mL^{-1}. The quantification was performed by representing the peak obtained by MS analysis versus the peptide concentration. Plots were made with the peak area of the molecular ions with m/z value, corresponding to parental ion, and their sodium and potassium adducts. Five calibration points were obtained from 1 to 16 μg mL^{-1}. Linear ($y = a + bx$) regression for the calibration curves was estimated. The following curves were obtained (a) $y = 1.40 \times 10^6 x - 1.92 \times 10^6$ ($R^2 = 0.997$) for AYFYPEL; (b) $y = 2.14 \times 10^6 x - 1.27 \times 10^6$ ($R^2 = 0.998$) for YFYPEL; (c) $y = 6.59 \times 10^6 x + 2.49 \times 10^6$ ($R^2 = 0.995$) for RYLGY, and (d) $y = 2.21 \times 10^6 x - 1.75 \times 10^6$ ($R^2 = 0.999$) for YQKFPQY.

To study peptide stability during experiments, cell supernatants were also analysed by RP-HPLC-MS/MS using a reverse phase Mediterranea Sea C18 Column (150 × 2.1 mm i.d., 5 μm particle size)

(Teknokroma, Barcelona, Spain). The samples were eluted at 0.2 mL min^{-1}.

Data obtained were processed and transformed to spectra representing mass values using the Data Analysis program (version 4.0, Bruker Daltonik). To process the MS/MS spectra and to perform peptide sequencing BioTools (version 3.1, Bruker Daltonik) was used.

2.6. Statistical analysis

Data were analysed by a two-way analysis of variance (ANOVA), followed by the Bonferroni test. For a better comparison of the concentrations versus control data for each time, data were analysed by a one-way ANOVA, followed by the Newman–Keuls test. GraphPad Prism 4 software was used to find significant differences between means and controls as $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

3. Results

3.1. Effect of synthetic peptides on MUC5AC expression and mucin secretion by HT29-MTX cells

HT29-MTX cells were exposed to three concentrations (0.5, 0.1, and 0.05 mM) of casein-derived peptides which have structures that fit the requirements to bind opioid receptors, i.e., RYLGY, AYFYPEL, YFYPEL, and YQKFPQY. These peptides were previously identified in a peptic casein hydrolysate with proven antihypertensive activity (Contreras et al., 2009). The level of MUC5AC mRNA was followed by qPCR up to 24 h (Fig. 1). Peptides AYFYPEL and YFYPEL produced an increase in the relative expression of the gene that reached the highest level after 4 h of exposure. The maximal response was 1.79-fold basal level ($P < 0.01$) at the highest concentration (0.5 mM) of YFYPEL. The homologous peptide, AYFYPEL, showed MUC5AC increased expression at 0.1 mM at 4 h (1.74-fold basal level, $P < 0.05$). Peptide RYLGY showed expression values over 1.7-fold basal level, although due to the high variability, it did not reach significance. The sequence YQKFPQY did not elicit a substantial change in the level of expression of MUC5AC.

Mucin secretion was determined in the supernatants of the cells submitted to the treatment with AYFYPEL and YFYPEL, where a significant increase in MUC5AC expression was found. The maximal percentages of mucin-like glycoprotein were reached at 4 h with 162% of control ($P < 0.05$) for AYFYPEL and 166% ($P < 0.01$) for YFYPEL (Fig. 2), which also showed a significant increase of mucin secretion after 2 h of exposure (152%; $P < 0.05$).

3.2. Determination of the stability of peptides in the HT29-MTX cell culture

Peptide stability during treatment was studied by RP-HPLC-MS/MS analysis of the cell supernatants. This analysis aimed to estimate the degradation of the parent peptide by the action of the cellular peptidases and allowed the identification of the released peptide fragments. Synthetic peptides were mostly stable up to 8 h of treatment and only small amounts of peptide fragments were detected (Fig. 3). In general, an appreciable decrease of the synthetic peptide concentration in the medium was observed between 8 and 24 h, although it was not always in accordance with the release of the derived fragments. In addition to hydrolysis, the reduced peptide presence with incubation time could be also attributed to its localisation in the mucin layer or intracellularly. Peptide YFYPEL was hydrolysed to FYPEL, but 8 h after exposure, slight degradation was found (15% of the 2 h value), and a further YFYPEL decrease was only partially reflected in FYPEL occurrence. Interestingly, from peptide AYFYPEL, the active peptide YFYPEL was formed besides FYPEL, but at too low rate to be considered responsible for the observed activity. Both RYLGY and YQKFPQY were similarly stable for the first 8 h, giving a unique derived fragment, YLGY and pyroglutamic acid derivative of parental peptide (m/z 793.4), respectively.

3.3. Effect of casein hydrolysate on MUC5AC expression and mucin secretion

Given the effect on MUC5AC expression and mucin secretion shown by the two contained peptides AYFYPEL and YFYPEL, the casein hydrolysate was assayed at two different concentrations

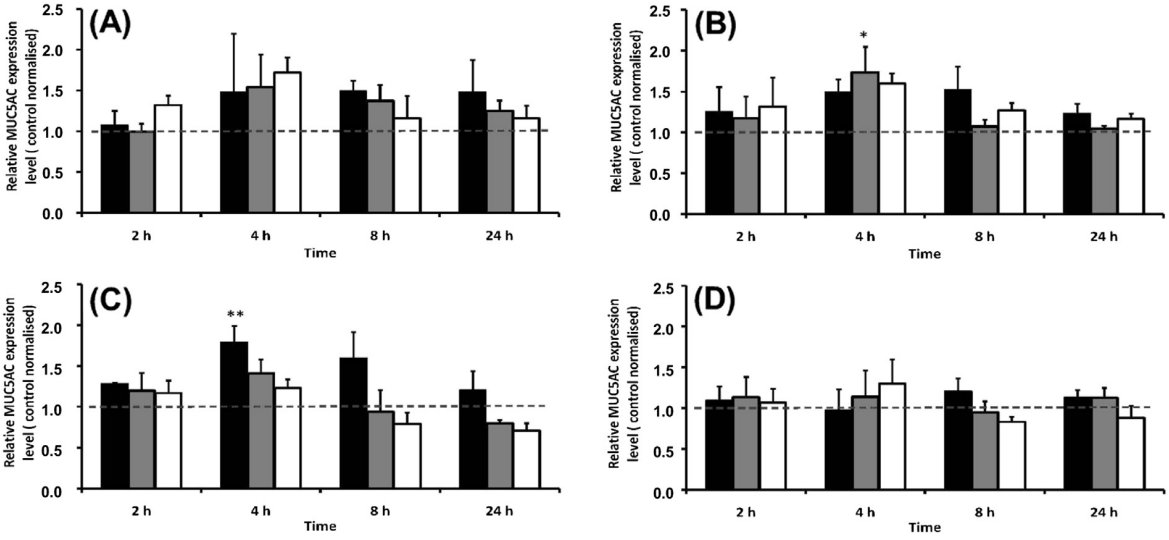

Fig. 1. Time course effect at three different concentrations (□, 0.05 mM; ▩, 0.1 mM; ■, 0.5 mM) of α_{S1}-casein fragments 90–94 RYLGY (A), 143–149 AYFYPEL (B), 144–149 YFYPEL (C), and α_{S2}-casein fragment 89–95 YQKFPQY (D), on MUC5AC mRNA level in HT29-MTX cells determined by quantitative RT-PCR. Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophilin as reference gene. Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by two-way ANOVA applying the Bonferroni test: (*) $P < 0.05$, (**) $P < 0.01$.

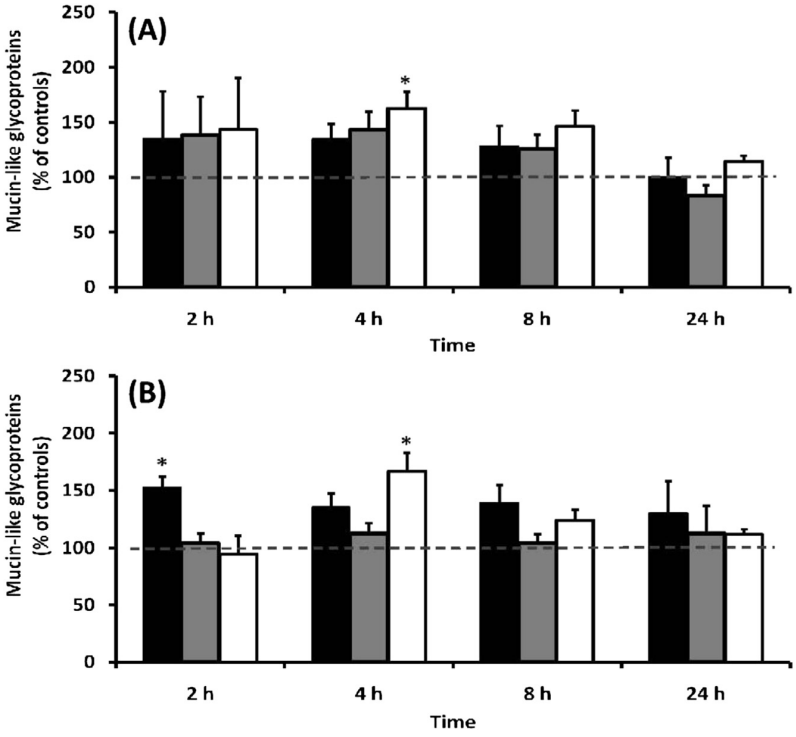

Fig. 2. Time course effect at three different concentrations (□, 0.05 mM; ▒, 0.1 mM; ■, 0.5 mM) of α_{S1}-casein fragments 143–149 AYFYPEL (A), and 144–149 YFYPEL (B), on mucin secretion in HT29-MTX cells determined by enzyme-linked lectin assay. Data are expressed as mucin-like glycoprotein secretion as a percentage of control (untreated cells). Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by one-way ANOVA applying the Newman–Keuls test: (*) $P < 0.05$.

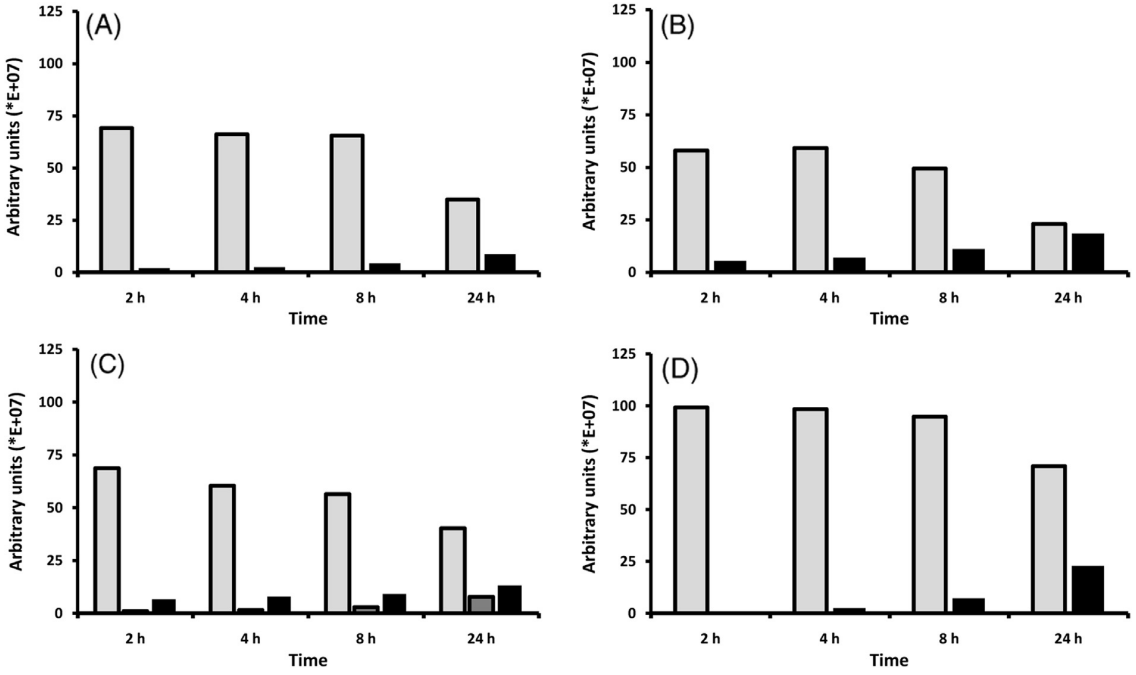

Fig. 3. Time course stability at 0.5 mM of: (A) α_{S1}-casein fragment 90–94 RYLGY (▒) and derivative YLGY (■); (B) α_{S1}-casein fragment 144–149 YFYPEL (▒) and derivative FYPEL (■); (C) α_{S1}-casein fragment 143–149 AYFYPEL (▒) and derivatives YFYPEL (▒) and FYPEL (■); (D) α_{S2}-casein fragment 89-95 YQKFPQY (▒) and the unidentified peptide derivative of m/z 793.4 (■) in cell supernatants as determined by reverse phase high performance liquid chromatography and tandem mass spectrometry. Arbitrary units expressed as the peak area of the molecular ions with m/z value, corresponding to parental ion and their sodium and potassium adducts.

(0.1 and 1%) during 2, 4, 8, and 24 h (Fig. 4). The level of MUC5AC expression was significantly increased at 4 h, (1.8-fold basal level) when hydrolysate concentrations of 0.1% and 1.0% were tested. The time of maximum expression, 4 h, was the same as that found for the synthetic peptides AYFYPEL and YFYPEL. When mucin secretion was determined, the highest response was found at 8 h with the 0.1% hydrolysate (210% of controls, $P < 0.001$) and secretion at 24 h was also significantly enhanced (172% of controls, $P < 0.01$). It was not possible to perform the mucin determination in the supernatants containing 1% hydrolysate probably due to the interference of peptides or other components at high concentration with the ELLA reagents.

The quantification in the hydrolysate of the four contained peptides with probable ability to interact with opioid receptors was determined by RP-HPLC-MS/MS and a similar abundance for all of them was found (1.93 mg of RYLGY, 4.01 mg AYFYPEL, 2.61 mg of YFYPEL, and 2.77 mg of YQKFPQY per g of hydrolysate). These values imply concentrations of the peptides in the 1% (w/v) hydrolysate of 0.029 mM RYLGY, 0.044 mM AYFYPEL, 0.031 mM YFYPEL, and 0.028 mM YQKFPQY, i.e., slightly lower than the lowest dose assayed for the synthetic peptides individually (0.05 mM).

4. Discussion

This work provides evidence that casein-derived peptides different from the β-casein fragments, previously described, have a stimulatory effect on mucin secretion and gene expression in human intestinal cells. All assayed synthetic peptides share a common structure favourable to binding to opioid receptors, i.e., the presence of a Tyr in the first or second position from the N-terminal end and an aromatic residue at the third and/or fourth position. The opioid activity of RYLGYL and RYLGYLE had been previously demonstrated by Loukas et al. (1983) but, to the best of our knowledge, the sequences here tested had not been previously described as opioid peptides. The possible interaction of these sequences with μ- or δ-opioid receptors undoubtedly merits further research and experiments with guinea pig ileum preparations are already in progress.

From the RT-PCR experiments, sequences AYFYPEL and YFYPEL were identified as those more active on MUC5AC expression and both peptides reached similar expression values as those previously described for β-casomorphin 7 (Zoghbi et al., 2006) and α-lactorphin (Martínez-Maqueda et al., 2012). Regarding the time at which the maximum effect was found, the time-course experiments showed a significant increase of MUC5AC mRNA levels at 4 h. This time is shorter than that previously reported for β-casormorphin-7, for which the maximal response was found after 24 h of treatment (Zoghbi et al., 2006). Previous results obtained in our laboratory with α-lactorphin showed that mucin discharge preceded the increase of MUC5AC expression, probably to replenish the intracellular mucin pool in goblet cells (Martínez-Maqueda et al., 2012). In the present study, this behaviour could be observed for YFYPEL with an increased mucin concentration at 2 h and a maximum expression level at 4 h. However for AYFYPEL, maximum mucin secretion and MUC5AC expression occurred at 4 h, simultaneously.

Interestingly, peptide AYFYPEL was partially degraded to the active form YFYPEL by the action of cellular peptidases although the amount released at 4 h does not allow anticipation of a significant

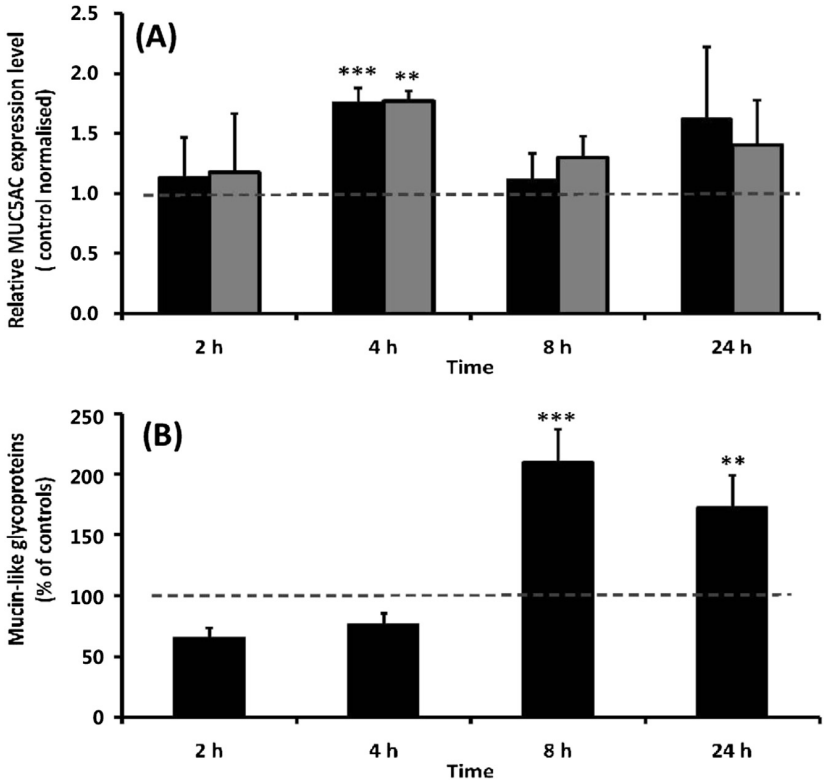

Fig. 4. Time-course effect of the casein hydrolysate in HT29-MTX cells. A) MUC5AC mRNA level determined by quantitative RT-PCR after addition of the hydrolysate at two different concentrations (■, 0.1%, w/v; ■, 1%, w/v). Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophillin as reference gene. Significant differences of each concentration versus control were determined by one-way ANOVA applying the Newman–Keuls test. B) Mucin secretion determined by enzyme-linked lectin assay after addition of the casein hydrolysate at the concentration of 0.1% (w/v). Data are expressed as mucin-like glycoprotein secretion as a percentage of control (untreated cells). Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by two-way ANOVA applying the Bonferroni test: (**) $P < 0.01$; (***) $P < 0.001$.

contribution of the peptide fragment to the observed activity. When testing the casein hydrolysate, the increased secretion was found later than the significant stimulation of the gene expression at 4 h although the relative MUC5AC expression also increased at 24 h without reaching significance. It is noteworthy that the hydrolysate effect on mucin secretion was higher than that found for the peptides AYFYPEL and YFYPEL separately (210% compared with 160% of controls, approximately). Moreover, the times of maximum secretion in the 0.1% hydrolysate treatment (8 and 24 h) were delayed compared with those observed with AYFYPEL (4 h) and YFYPEL effect (2 and 4 h). Based on the peptide quantification, the concentration of active peptides in the 0.1% hydrolysate was ten-fold lower than that of the synthetic peptides that have separately shown activity on mucin secretion.

In addition to the contribution of AYFYPEL and YFYPEL, these results suggest that peptides or other components of the hydrolysate could participate in a synergistic manner or through a non-opioid mechanism. Alternatively, the action of cellular peptidases may produce the release of new peptides, although supernatant analysis of synthetic peptides shows that synthetic peptides remained stable up to 8 h. To clarify if opioid receptors are involved in the mucin production activity, experiments using an opioid antagonist (ciprodime) are being conducted. In any case, the clear effect of the casein hydrolysate on mucin secretion and MUC5AC expression is consistent with a previous report by Han, Deglaire, Sengupta, and Moughan (2008). Here it was shown that a casein hydrolysate produced a significant increase of Muc3 mRNA levels in the small intestine tissue and Muc4 gene expression in the colon tissue of rats fed with the hydrolysate for 8 days. In contrast, no effect was found with a free L-amino acid diet simulating the hydrolysate (Han et al., 2008).

There are obvious limitations with the use of a cell culture model in this study. Although intestinal cell lines constitute a suitable approximation to the in vivo environment, certain shortcomings may be present as non-identical natural responses or physiology in comparison with the intestine (Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011). General limitations of cancer-derived lines are non-specificity, altered glycosylation and unresponsiveness to hormones or cytokines (Peracaula, Barrabes, Sarrats, Rudd, & de Llorens, 2008). The HT29-MTX cell line is a mucin-secreting cell line that forms a homogeneous monolayer of polarised goblet cells that exhibit a discrete apical brush border. This cell culture has proven to be a reliable tool for the study of gastrointestinal mucin secretion (Lesuffleur et al., 1993; Zoghbi et al., 2006) but results should be confirmed by animal testing.

It is important to highlight that some of the peptides here tested had been previously found in the human gastric and intestinal contents after milk ingestion or in vitro gastrointestinal simulations of dairy products. This is the case for AYFYPEL, which is released in the human stomach after milk or yoghurt ingestion and the shorter form, YFYPEL that was detected in duodenal samples after milk ingestion (Chabance et al., 1998). Similarly, these two peptides have also been found in simulated gastrointestinal digestions of infant formulae, milk and yoghurt by different authors (Dupont et al., 2010; Hernández-Ledesma, Quirós, Amigo, & Recio, 2007). The doses of synthetic peptides used in our study are within the concentration range theoretically expected in the intestinal contents after milk ingestion.

5. Conclusions

Some casein-derived peptides whose sequences might anticipate interaction with opioid receptors have been assayed in HT29-MTX cells to study their influence on mucin production. Two of them, AYFYPEL and YFYPEL, have proven to significantly increase MUC5AC gene expression and mucin secretion. A casein hydrolysate, containing these peptides among the most abundant species, is able to stimulate MUC5AC expression and mucin secretion at a higher rate than the individual peptides. Thus, a casein hydrolysate and two contained peptides, AYFYPEL and YFYPEL with probable ability to bind opioid receptors, have demonstrated their ability to affect mucin secretion and MUC5AC expression in human HT29-MTX cells. Such knowledge may help the development of functional foods that promote the strengthening of the mucus barrier and its protective role in gastrointestinal diseases.

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Mucin stimulatory activity of α_{S1}-casein f(143-149)-derived peptides. Novel casein fragments with opioid activity.

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ABSTRACT

The specific effect of some peptides and protein hydrolysates on intestinal mucus production has been attributed to the activation of μ-opioid receptors that are present in the membrane of intestinal goblet cells. Our group has previously observed a potent mucin stimulatory activity for two α_{s1}-casein peptides with sequences AYFYPEL and YFYPEL. The aim of this work was to assess the minimum active fragment from this α_{s1}-casein-derived peptide able to show mucin production activity in HT29-MTX intestinal human cells and to evaluate if this activity was mediated through the interaction with μ-opioid receptors. Shorter peptide forms, YFYPE and YFYP, did not exhibit significant over-expression of MUC5AC, the major secreted mucin gene in this cell line, compared with controls (untreated cells). Therefore, peptide YFYPEL is proposed as the minimum fragment with effect on mucin synthesis. However, experiments in goblet cells using cyprodime as opioid antagonist failed to demonstrate that the μ-opioid pathway is involved in the mucin stimulatory activity of YFYPEL. In addition, AYFYPEL and its derived fragments were tested by guinea-pig ileum assays to evaluate the opioid activity of these peptides. Fragments YFYPEL and YFYPE have shown, for the first time, inhibition of the electrically induced contractions in guinea-pig ileum preparations, and thus, it demonstrates the interaction of these peptides with opioid receptors. Consequently, the existence of additional pathways should be considered in the effect on mucin production.

KEYWORDS: Mucin, goblet cells, casein, opioid peptides, guinea-pig ileum

1. INTRODUCTION

The gastrointestinal mucus gel covering the mucosal surface is a major component of physiological defense mechanisms. Many studies support the hypothesis that alterations in mucin synthesis, secretion, and/or degradation may be involved in the initiation or maintenance of intestinal diseases (Einerhand et al., 2002). In this context, the strengthening of the mucus gel, in particular by nutrients, could be extremely beneficial. Many studies carried out *in vivo* or *in vitro* have shown that dietary fibers, short-fatty acids and probiotics can modify the dynamics of mucus by increasing the secretion or expression of mucins or even the number of goblet cells (Ito et al., 2009; Gaudier et al., 2004; Burger van Paassen, 2009). The specific effect of proteins and peptides on intestinal mucus production was first studied using an isolated vascularly perfused rat jejunum preparation. Luminal administration of a casein hydrolysate at physiological level stimulated mucin secretion as much as 4-fold. A lactalbumin hydrolysate also stimulated mucin discharge whereas egg albumin or meat hydrolysates, as well as native proteins, failed to induce a statistically significant increase in mucin production (Claustre et al., 2002). Interestingly, the opioid peptide β-casomorphin-7 gave rise to a sharp increase in mucin discharge (Trompette et al. 2003). All the effects were inhibited by naloxone (an opioid antagonist), which supports the hypothesis that the effect is mediated by opioid-receptor activation.

The prominent localization of opioid receptors in the gut is the myenteric and submucosal plexus. In is interesting to note, however, that, the presence of μ-opioid receptors has also been demonstrated on epithelial cells of the rat, pig, and guinea-pig (Lang et al., 1996; Nano et al., 2000; Quito et al., 1991), suggesting that opioid agonists may act directly on the intestinal epithelium to regulate its functions. The exposure of human (HT29-MTX) and rat (DHE) intestinal mucus-secreting cells to β-casomorphin-7 raised significantly the secreted mucin and the mRNA level of the major secreted mucin genes (Zoghbi et al., 2006).

Recently, other food-derived peptides have shown activity on mucin secretion and MUC5AC expression in human cells (Martínez-Maqueda et al., 2012; 2013a). Among the peptides showing this activity, α-lactorfin and β-lactorfin had been previously reported as opioid peptides. However, two fragments from bovine α_{s1}-casein, AYFYPEL (143-149) and YFYPEL (144-149), not previously described with this activity, showed an important effect on mucus production by increasing both mucin secretion and MUC5AC gene expression (Martínez-Maqueda et al., 2013b).The sequences of these peptides fit the reported structure to interact with opioid receptors.

The objective of this work was to assess the minimum fragment from AYFYPEL able to show mucin secretory activity in human cells. To investigate if the proposed opioid mechanism is involved, cell culture experiments using an opioid antagonist have been carried out and the opioid activity of all sequences has been tested by guinea-pig ileum assays.

2. MATERIALS AND METHODS

2.1. Peptides

α_{s1}-casein fragments 143-149 (AYFYPEL), 144-149 (YFYPEL), 144-148 (YFYPE) and β-casomorphin (YPFPGPI), were synthesized using conventional solid-phase FMOC synthesis with a 433A peptide synthesizer (Applied Biosystems, Warrington, UK). Their purity (>90%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry.α_{s1}-casein fragment 144-147 (YFYP) was synthesized by Genscript (Piscataway, NJ, USA).

2.2. Cell culture

HT29-MTX, a human colon adenocarcinoma-derived mucin-secreting goblet cell line was provided by Dr. Thécla Lesuffleur (Lessufleur et al., 1993). The cell line was grown in plastic 75-cm^2 culture flasks in DMEM supplemented with 10% FBS and 10 mL/L penicillin-streptomycin solution (all from Gibco, Paisley, UK) at 37ºC in a 5% CO_2 atmosphere in a humidified incubator. Cells were passaged weekly using trypsin/EDTA 0.05% (Gibco). The culture medium was changed every two days. To study the effect of peptides, cells were seeded at a density of 5×10^5 cells per well in 12-well culture plates (Nunc, Roskilde, Denmark). The cell line was used between passages 12 and 19. Experiments were performed 21 days after confluency. Twenty-four hours before the studies, the culture medium was replaced by serum- and antibiotic-free medium to starve the cells and to eliminate any interference from extraneous proteins or hormones. After serum-free medium removal, the monolayer was washed twice with PBS. Serum-free medium with or without peptide (0.05-0.5mM) and/or the addition of cyprodime (0.01mM) was added to the cells and incubated at 37ºC for 10 min to 24 h in a 5% CO_2 humidified atmosphere. The supernatants were collected, frozen and stored at -70ºC. The total RNA was isolated with Nucleospin® RNA II (Macherey-Nagel, Düren, Germany).

2.3Quantitative RT-PCR assays (qRT-PCR)

Quantitative RT-PCR amplification was carried out using a Lightcycler 480 (Roche, Mannheim, Germany) in 384 wells microplates (Roche). RNA (375 ng) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer´s instruction. For MUC5AC (accession no. AJ001402), target gene, primers 2870-2889/3109-3091 were used. For reference genes cyclophillin (accession no. Y00052) and β-actin (accession no. NM_001101) primers 280-340/445-421 and 879-896/1076-1053, respectively, were used (Zoghbi et al., 2006; Tai, Wong, Lam, Wu, Yu, Koo & Cho, 2008). The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 5 µL 2x SYBR Green real-time PCR Master Mix (Roche) 0.25 µL of a 10 µM of gene-specific forward and reverse primers, 0.27 µL of cDNA and 4.23 µL of water. Amplification was initiated at 95ºC for 5 min, followed by 45 cycles of 95ºC for 10 s, 60ºC for 10 s and 72ºC for 10 s. Control PCRs were included to confirm the absence of primer dimer formation (no-template control), and to verify that there was no DNA contamination (without RT enzyme negative control). All real-time PCR assays amplified a single product as determined by melting curve analysis.

The relative expression levels of the target gene were calculated using the comparative critical threshold method (ΔΔCt). Human cyclophilin and β-actin were tested as reference gene. Cyclophilin gene was chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used. All experiments were performed at last three times in triplicate.

2.5 In vitro isolated preparation

Female guinea-pigs weighing 300-450 g were used. Myenteric plexus-longitudinal muscle strips (MP-LM) were isolated from guinea-pig ileum as described by Ambache (1954).Tissues were suspended in a 10 ml organ bath containing 5 ml of Krebs solution (NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11mM). This solution was continuously gassed with 95% O_2 and 5% CO_2. Tissues were kept under 1g of resting tension, at 37ºC and were electrically stimulated through two platinum ring electrodes. MP-LM strips were continuously stimulated with rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency. The isometric force was monitored by computer using a MacLab data recording and analysis system. The agonistic activity of the peptides was evaluated performing cumulative concentration-response curves (6.1×10^{-8} –10^{-5} M) that were constructed in a step by step manner as follows: peptides were added to the organ bath 15 minutes after the beginning of

electrical stimulation and their effect on the electrically induced contractions was evaluated until the response had reached a plateau. The interval between applications of increasing concentrations was 9 min. Results were expressed as % of inhibition, taking the mean amplitude of the last five contractions before the addition of the peptides as 100%. Each tissue was employed only once.

To corroborate that the inhibitory effect of the peptides was mediated through selective interaction with opioid receptors, one dose of naloxone (10^{-6} M), an opioid antagonist, was added to the organ bath at the end of each experiment.

2.6 Analysis by HPLC-tandem mass spectrometry

Analysis of the peptides in organ bath solution or in Krebs solution were performed using an Acquity liquid chromatography system (Waters) connected to microToF II Quadrupole-Time-of-Flight mass spectrometer (Bruker). A Poroshell C18 column (100 mm length, 2.1 mm internal diameter, 1.7 µm packing) was used. A binary elution gradient, based on water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (v/v), and operated at a flow rate of 0.2 mL/min, was adopted. A gradient elution program based on solvent B increase from 0% to 70%in 30 min was used. Column reconditioning at the initial mobile phase composition was accomplished in 15 minutes. The Acquity UPLC Autosampler (Waters) was adopted for chromatographic injections (injection volume: 10 µL).

ESI-MS acquisitions were carried out in positive ion mode. The LC-MS system was controlled by the HyStar 3.2 software (Bruker). The main parameters of the ESI interface and of the quadrupole were optimized preliminarily by analyzing the tryptic digests of standard caseinate solutions and the following values were finally adopted: Dry temperature 180°C; Dry gas flow (L/min) 4.0; Nebulizer gas pressure (Bar) 0.4; Capillary voltage 4.5 kV; Quadrupole Ion Energy (eV) 5.0. The following values were adopted for the main ToF parameters: Repetition Rate 5.000 kHz, Flight Tube -8600 V, Reflector +1680V. The ToF analyzer was calibrated on a daily basis in MS mode, using the m/z ratios of adduct ions arising from sodium formiate as reference.

3. RESULTS AND DISCUSSION

3.1. Activity of bovine α_{s1}-casein f144-148 (YFYPE) and f144-147 (YFYP) on MUC5AC expression

In order to find the minimal active fragment from bovine α_{s1}-caseinAYFYPEL and a likely structure-activity relationship involved in the mucin production activity, the effect of two derived sequences, YFYPE and YFYP, was studied. As the parental sequences, YFYPE and YFYP structures satisfy the reported requirement to present probable

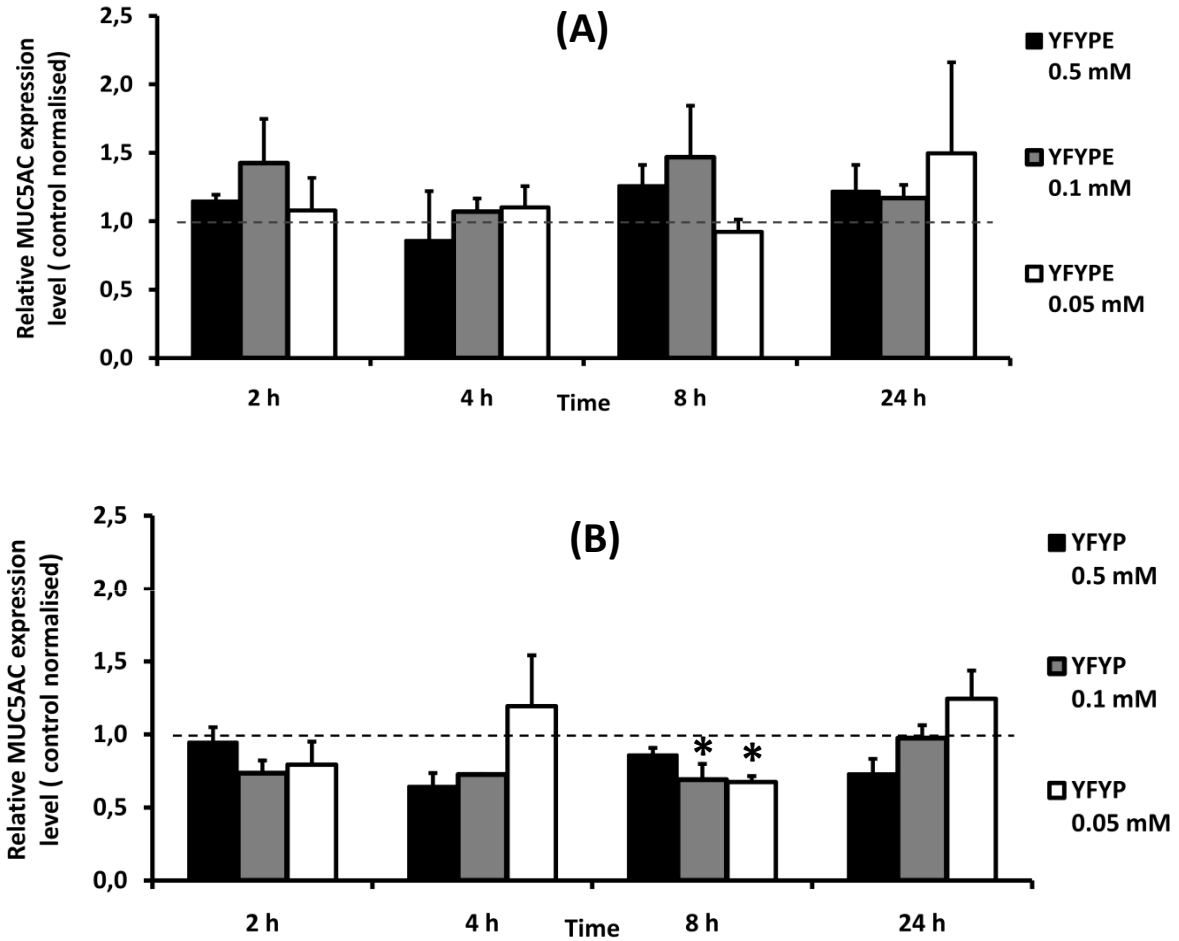

Figure 1. Time course effect at three different concentrations (0.05, 0.1, and 0.5 mM) of α_{s1}-casein fragments 144-148 YFYPE (A) and 144-147 YFYP (B), on MUC5AC mRNA level in HT29-MTX cells determined by quantitative RT-PCR. Data are expressed as relative MUC5AC expression level of control (untreated cells), using cyclophilin as reference gene. Each point represents the mean ± SE of three biological replicates in triplicate. Significant differences of each concentration versus control were determined by one-way ANOVA applying the Newman-Keuls test: (∗) $P < 0.05$.

opioid activity, i.e., the presence of a tyrosine in the N-terminal end position and another aromatic residue in the third and/or fourth position (Meisel et al., 1998).
The relative expression of MUC5AC was determined by qRT-PCR after exposure of HT29-MTX cells to the peptides action. Figure 1 shows the time-course effect on the relative MUC5AC expression level along 24 h of treatment with YFYPE and YFYP at three concentrations (0.5, 0.1 and 0.05 mM). No significant MUC5AC over-expression was observed compared with controls (untreated cells). Figure 1A represents a tendency towards increased MUC5AC transcription after treatment with YFYPE, reaching maximal values around 1.5 fold-basal level of expression at 2, 8 and 24 h, but no statistical differences were found due to elevated biological variability. Figure 1B shows the relative MUC5AC mRNA levels measured after treatment with YFYP, where most of mean values are below basal line, diminishing up to 0.65 fold-basal level. Significant reductions in MUC5AC expression ($P < 0.05$) resulted in YFYP treatment at 8 h for 0.1 and 0.05 mM concentrations with values of 0.69 and 0.67 fold-basal level. According to the results, YFYPE and YFYP exhibit apparently different behaviours on the mucin gene expression in HT29-MTX cells.

Table 1 summarizes the maximal YFYPE and YFYP values of MUC5AC relative expression and compares with previously reported results for AYFYPEL and YFYPEL. As it can be appreciated, the time point of 4 h shows the highest difference on mucin gene expression level between peptides with or without significant activity. At this time, YFYPE and YFYP, just reached 1.10 and 1.19 fold basal level without statistical significance. At other incubation times, YFYPE achieved similar of higher MUC5AC mRNA levels than those found for AYFYPEL and YFYPEL (1.43 vs 1.31/1.28 and 1.50 vs 1.23/1.20 fold basal level, respectively), but these values were not statistically different than the control. Based on these results, it could be stated that YFYPEL represents the minimum active fragment on MUC5AC gene expression.

With the purpose of clarifying if the reported activity of YFYPEL on mucin production is mediated by opioid receptors, cyprodime, a μ-opioid antagonist, was employed. Individually or together with YFYPEL, the addition of cyprodime did not provoke any significant change on MUC5AC mRNA levels compared with those observed in controls and YFYPEL treatment, respectively (data not shown). On this basis, the mechanism via μ-opioid receptors may be discarded, at least for YFYPEL activity, although other types of opioid receptors, δ or κ, could participate. Previously, Zoghbi et al. (2006) reported that the activity of β-casomorphin 7 on mucin production in HT29-MTX cells occurred via μ-opioid interactions, because the effect was prevented by

Table 1: Maximal MUC5AC expression level as relative of control (untreated cells) at concentrations between 0.05 and 0.5 mM in HT29-MTX cells, determined by quantitative RT-PCR. Each point represents the mean of three biological replicates in triplicate. Significant differences versus control were determined by two-way ANOVA applying the Bonferroni test: (∗) $P < 0.05$; (∗∗) $P < 0.01$.

	AYFYPEL	YFYPEL	YFYPE	YFYP
2h	1.31	1.28	1.43	0.95
4h	***1.74****	***1.79****	1.10	1.19
8h	1.53	1.60	1.47	0.86
24h	1.23	1.20	1.50	1.24

pretreatment with cyprodime. In contrast, a recent work has described that the activity of the β-casein 94-123 fragment on mucin secretion and MUC2 and MUC4 expression in the identical cell line was not modified by cyprodime action (Plaisancié et al., 2013). It is obvious that further inhibition studies are necessary, using additional opioid antagonists, such as, naloxone which blocks the 3 opioid receptors subtypes, to investigate the mechanism involved in the effect on mucin production.

3.2. Activity of α_{s1}-casein f143-149 (AYFYPEL) and derived peptides on guinea-pig ileum preparations

Figure 2 shows the dose-response inhibition of the electrically induced contractions in MP-LM preparations when β-casomorphin and α_{s1}-casein fragment 143-149 (AYFYPEL) were assayed. As expected, β-casomorphin induced a dose-dependent inhibition up to the 5×10^{-6} M concentration, where 20.1 ± 4.7% of inhibition was reached. The decrease observed at the 10^{-5} M point could be attributed to a lack of receptor specificity at this peptide concentration (Figure 2A). AYFYPEL showed an increased inhibition between the 10^{-7} and 5×10^{-7} M concentrations reaching 8.4 ± 4.1% but the values did not show any further increase (Figure 2B).The *in vitro* effect of both peptides was completely reversed by the administration of naloxone.

As 10^{-5} M was considered a too high concentration to achieve specific response of the opioid receptors, cumulative curves with maximum dose of 5×10^{-6}M were used in next experiments. Figure 3 shows the dose-response inhibitions induced when the AYFYPEL-derived fragments were assayed. All peptides showed maximal average response at 5.5×10^{-7} M and lower values were encountered at higher concentrations. In the case of YFYPEL, a maximum response of 25.5± 7.2% was reached followed by a smooth decrease. YFYPE reached a mean value of 30.9 ± 4.1% followed by a steep decrease. YFYP showed no increased inhibition. The effect of YFYPEL and YFYPE was reversed by naloxone but that of YFYP was partially reversed, which indicated the non-opioid behaviour of this peptide. The results show opioid activity for the sequences YFYPEL and YFYPE for the first time, with percentages of inhibition higher than that of β-casomorphin 7.

In order to check the peptide stability during the experiments, aliquots of the organ bath were withdrawn and analysed by RP-HPLC-MS/MS. The chromatographic areas of 5 x 10^{-6} M treatment samples and their references, i.e., the corresponding peptide dissolved in control Krebs solution at the same concentration, were determined. β-casomorphin 7 kept similar area (82.07%) respect to that found for its reference, in accordance to the reported resistance of this peptide to enzymatic hydrolysis (Lee and

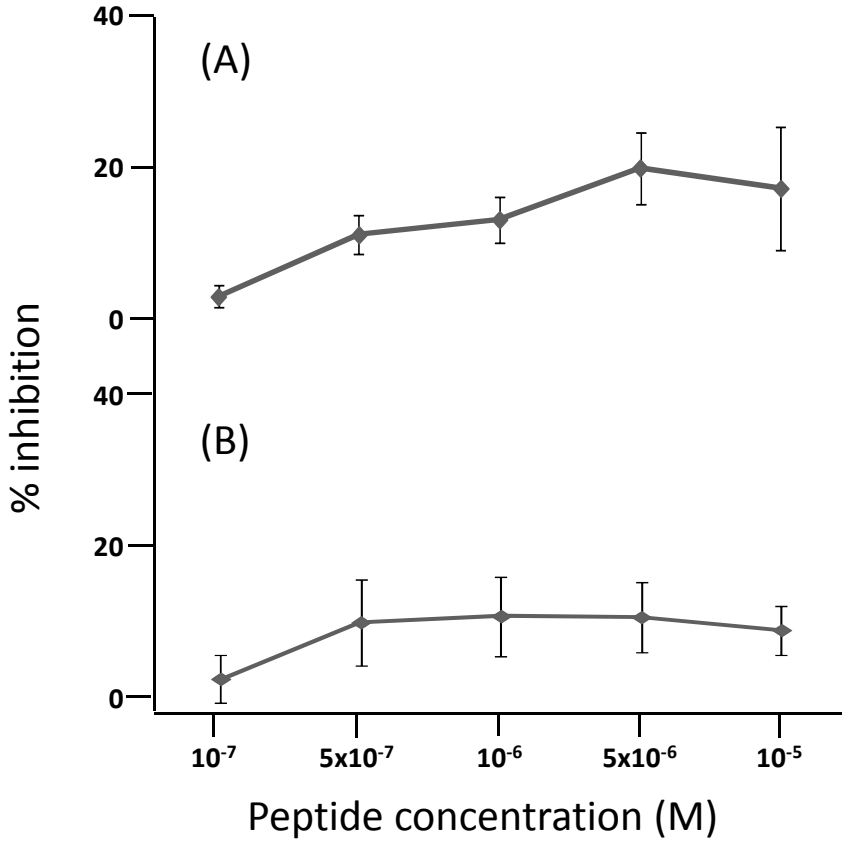

Figure 2. Inhibition of the electrically induced contractions in guinea pig ileum preparations at increasing concentrations (cumulative curve) of β-casomorphin 7 (A) and α_{s1}-casein fragment 143-149 AYFYPEL (B). Each point represents the mean % ± SEM (n=6).

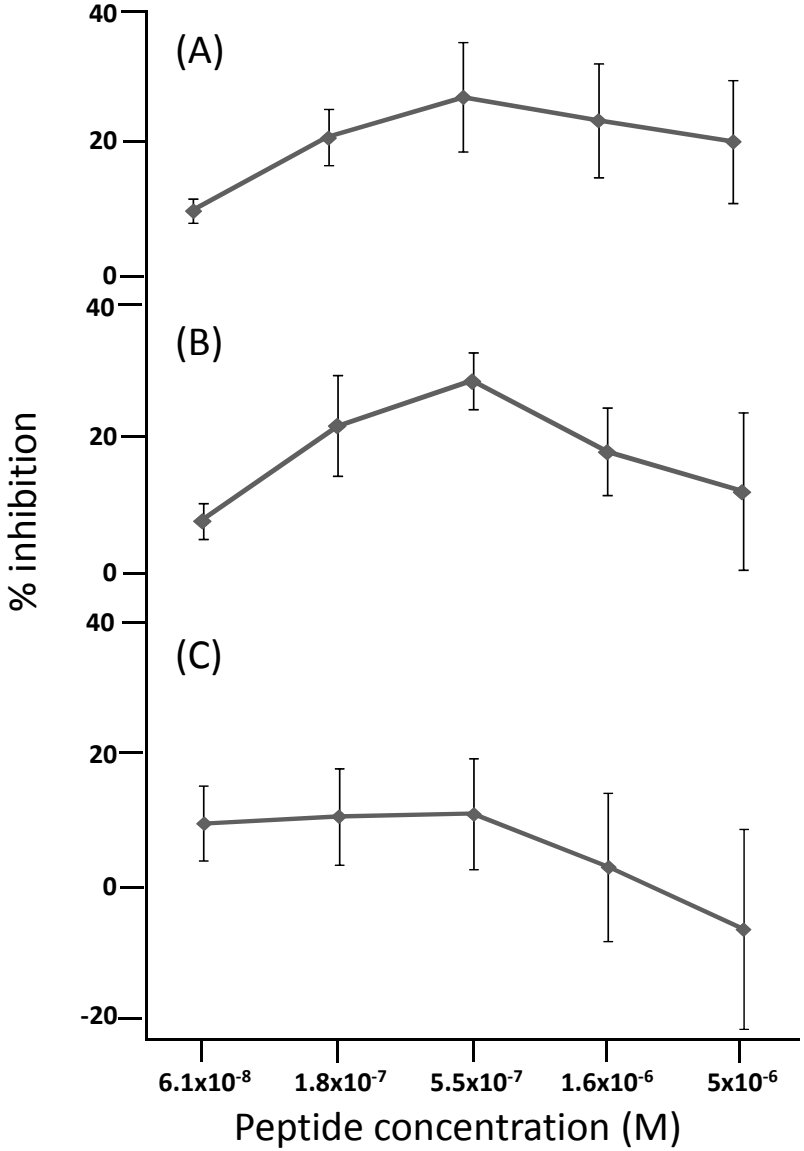

Figure 3. Inhibition of the electrically induced contractions in guinea pig ileum preparations at increasing concentrations (cumulative curve) of α_{s1}-casein fragment 144-149 YFYPEL (A), α_{s1}-casein fragment 144-148 YFYPE (B) and α_{s1}-casein fragment 144-147 YFYP (C). Each point represents the mean % ± SEM (n=6).

Lee, 2000). YFYPEL and YFYPE showed much smaller percentages from the reference (2.14% and 0.92%, respectively) in the withdrawn samples, with the appearance of FYPEL and YPE in the first case and FYPE in the second case, together with other unidentified peaks. In fact, their structure is similar to that of endogenous opioid peptides that are particularly sensitive to rapid hydrolysis by a number of peptidases that are present in the guinea-pig MP, as well as in other tissues (McKnight et al., 1983).

Opioid receptor ligands derived from milk proteins that exhibit naloxone inhibitable opioid activities, have been divided into two groups designated as “typical” and “atypical”. Typical peptides exhibit the definite N-terminal sequence YGGF of the endogenous opioid peptides: enkephalins, propiomelanocortin and prodynorphin. Atypical peptides are characterized by an N-terminal sequence Y-X-F or Y-X1-X2-F. The presence of tyrosine and an aromatic amino acid form a structural motif important in ligand-receptor binding (Teschemacher et al., 1997). β-casomorphins were the first identified and correspond to fragments of the β-casein sequence 60-70 (YPFPGPIPNSL) (Brantl et al., 1979). Three α-casein derived exorphins showing the YLGY sequence preceded or not by arginine are δ-selective receptor ligands (Loukas et al., 1983). Opioid peptides are also found encrypted within the primary sequence of whey proteins such as lactoferrin, α-lactalbumin, β-lactoglobulin and bovine serum albumin. The common structural feature among endogenous and exogenous opioid peptides is the presence of a tyrosine residue at the amino terminal end (except α-casein opioids) and the presence of another aromatic residue, e.g., phenylalanine of tyrosine, in the third or fourth position. The peptides here considered fit these motifs and were considered therefore candidates to show opioid activity.

The effect on mucin production by food protein hydrolysates and peptides appears to be mediated by more than one type of mechanism. The μ-opioid pathway plays an important role apparently, i.e., β-casomorphin 7, a reported casein-derived μ-opioid peptide, demonstrated activity in rat and human intestinal cell lines (Zoghbi et al., 2006), as well as in an ex vivo rat jejunum model (Claustre et al., 2002; Trompette et al., 2003), through activation of opioid receptors since the effects were inhibited by pretreatment with opioid antagonists. In contrast, a recent study has described that the activity of the β-casein 94-123 fragment on mucin release and expression of MUC2 and MUC4 in HT29-MTX was not prevented by the μ-opioid receptors blockage with cyprodime (Plaisancié et al., 2013). In a screening of several peptides with proved or probable μ- and δ-opioid activity, six out of eight peptides induced mucin secretion in

HT29-MTX cells (Martínez-Maqueda et al., 2012). Subsequently, the evaluation of the effect on MUC5AC expression by α-lactorphin and human β-casomorphin 5 showed that only the first provoked a significant increase, despite both had behaved as mucin secretors. In terms of opioid selectivity, results were not conclusive, i.e., the neocasomorphin, one of the peptides that did not induce mucin secretion, has higher μ-receptor affinity than α-lactorphin (Teschemacher et al., 1997). The influence of intensity of opioid interactions on mucin production activity is well characterized in the case of β-lactorphin, a β-lactoglobulin-derived peptide. In a previous study of our group, the amidated form of β-lactorphin (YLLF-NH2) demonstrated the ability of inducing MUC5AC expression and mucin secretion in HT29-MTX cells, whereas the non-amidated form just produced a lower increase of mucin secretion (Martínez-Maqueda et al., 2013a). Taking into account that the amidated form presents much higher opioid activity than the non-amidated form (Yoshikawa et al., 1986), the intensity of opioid interactions may explain the observed differences on mucin production effect. Interestingly, the results of opioid activity for AYFYPEL, YFYPEL and YFYPE, determined in this work by experiments with guinea-pig ileum preparations, do not match with the possibility of an exclusive μ-opioid mechanism in the mucin production enhancement because AYFYPEL hardly presented opioid affinity and showed a significant activity on mucin biosynthesis, in contrast to YFYPE that interacted noticeably with opioid receptors but did not produce any statistical increase of mucin gene expression. The possibility of additional mechanisms, different from opioid pathway, should be considered. Sprong et al. (2010) reported that the intake of a cheese whey protein rose mucin content in feces of rat with dextran sulfate sodium induced inflammation in the large intestine. The results were discussed according to the hypothesis of the threonine and cysteine action, supported by the lack of Muc2 over-expression and the obtaining of a comparable effect by casein supplemented in these amino acids. Threonine and cysteine constitute limiting amino acids in the mucin synthesis under intestinal inflammation conditions, and are abundant in whey proteins compared with other food proteins (Faure et al., 2006). Recently, two milk protein hydrolysates, one from whey protein concentrate enriched in β-lactoglobulin and the other one from commercial casein, have shown more potent effect on mucin production in HT29-MTX cells than that achieved separately by candidate peptides, on the basis of proved or probable opioid activity of them (Martínez-Maqueda et al., 2013a; 2013b). Thus, peptides or other components may contribute to the activity of both hydrolysates via a synergistic way or a non opioid mechanism. The presence of alternative pathways should be evaluated by additional studies.

In conclusion, the study of the MUC5AC expression in human cells treated with bovine α_{s1}-casein fragments derived from AYFYPEL has shown that YFYPEL is the minimum fragment able to stimulate mucin production. However, the concomitant use of cyprodime, an opioid antagonist, showed no effect on the activity. Therefore, it cannot be demonstrated that the opioid pathway is responsible for the stimulation, at least as the unique mechanism. Fragments YFYPEL and YFYPE showed for the first time inhibition of the electrically induced contractions in MP-LM preparations isolated from guinea-pig ileum although a dramatic decrease in the concentration of both peptides in the organ bath after some minutes has been observed. In order to confirm the novel opioid activity, experiments with protease inhibitors are already in progress.

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Antiulcerative activity of hydrolysates from β-lactoglobulin and casein

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Abstract

Peptic ulcers are a common disorder that occurs mainly in the stomach and the proximal duodenum. Several studies have shown the protective effect of dairy products, especially whey proteins and derived hydrolysates, against ulcerative lesions caused by ethanol, stress or indomethacin. The mucus strengthening represents an important mechanism in the defence of gastric mucosa. Previously, a hydrolysate from casein (CNH) and a hydrolysate from whey protein concentrate rich in β-lactoglobulin (WPH) demonstrated a stimulatory activity on mucus production in intestinal goblet cells. The aim of this work was to evaluate the possible antiulcerative activity of these two hydrolysates and commercial casein (CN). Antiulcerative properties were studied after acute administration of CN, CNH, and WPH at three doses in a model of ulcer induced by absolute ethanol in rats. All tested samples presented significant decreases in the ulcerative lesions index (ULI), compared with the saline solution, for the intermediate (300 mg kg-1 body weight (bw)) and the highest dose (1000 mg kg-1 bw), which reached decreases of 56.3-66.3% ULI. A dose-response relationship was found for both CNH and WPH. In order to evaluate if the observed antiulcerative activity was mediated by the action of sulfhydryl (SH) groups, an in vivo assay was performed by using pre-treatment with the inhibitor N-ethylmaleimide (NEM). The WPH antiulcerative activity was drastically decreased (from 41.3 to 9.2% ULI) when rats received NEM prior to acute administration of 500 mg kg-1 bw. In contrast, the CNH antiulcerative activity was not significantly affected by the blocking of SH groups. In conclusion, casein and β-lactoglobulin hydrolysates, in addition to casein, demonstrated antiulcerative properties in an ethanol-induced ulcer model and represent an alternative and promising therapy in the treatment of peptic ulcers.

Keywords: Peptides; Whey protein hydrolysate; Casein hydrolysate; Antiulcerative activity; Rat model

1. Introduction

Peptic ulcers are a common disorder that occurs mainly in the stomach and the proximal duodenum. A peptic ulcer results from an imbalance between aggressive factor(s) and cytoprotective factors, which include the function of the mucus-bicarbonate barrier, surface active phospholipids, prostaglandins (PGs), mucosal blood flow, cell renewal and migration, non-enzymatic and enzymatic antioxidants and some growth factors (Wallace & Granger, 1996).

It is now accepted that food is not only a supplier of nutrients but also a modulator of different physiological functions of the body. Because the gastrointestinal tract is in contact with food components and their digestion products, is the organ where this concept can be directly applied. In fact, many food components of different chemical nature have been found to exert modulation of intestinal functions (Shimizu, 2010), and some of them have demonstrated an antiulcerative effect. For instance, flavonoids may act in healing of gastric ulcers and they have been proposed as new alternatives for suppression or modulation of peptic ulcers associated with *Helicobacter pylori* (de Lira Mota et al., 2009). In addition to polyphenols, proteins and especially whey proteins and hydrolysates thereof have been found to exert antiulcerative properties. More rapid uptake of amino acids has been reported after the consumption of protein hydrolysates than intact proteins or free amino acids, being proposed are a better source of protein during repair of tissue damage (Thomson and Buckley, 2011). Several studies have shown the protective effect of α-lactalbumin against ulcerative lesions caused by ethanol, stress or indomethacin (Matsumoto et al., 2001; Mezzaroba et al., 2006). It was found that oral administration of this protein increased the level of PGE_2 and the mucin contents of both the gastric fluid and the adherent mucus gel layer (Ushida et al., 2003; Mezzaroba et al., 2006). This protein also caused PG-independent responses such as increase of the gastric luminal pH and fluid volume, and delay in gastric emptying (Ushida et al., 2003). More recently, it has been found that α-lactalbumin stimulates in cell cultures the

synthesis and secretion of mucins, the main macromolecular components of mucus, and the thickness of the mucus gel layer in rats, being this enhancing effect independent of endogenous PGE_2 (Ushida et al., 2007). Not only α-lactalbumin, but milk whey protein concentrates (WPC) or isolates (WPI) containing this protein have also demonstrated this protective effect against ulcerative lesions, and these effects were attributed to gastrin, sulfhydryl (SH) substances and some mechanisms related to mucus production (Rosaneli et al., 2004; Castro et al., 2010). The antiulcerative activity of these WPC was attributed to their α-lactalbumin content. On the contrary, no antiulcerative effect has been found for β-lactoglobulin (Matsumoto et al., 2001). More recently, a cheese WPC was found to protect against dextran sulphate sodium-induced colitis and the effect was attributed to its threonine and cysteine content given that these amino acids are limiting for mucin synthesis under chronic inflammatory bowel disease (Sprong et al., 2010). A WPC hydrolysed with extracts of *Cynara cardunculus* has demonstrated a protective effect against ulcerative lesions induced by ethanol. The protective activity of the hydrolysate was attributed to the stimulation of PGs and nitric oxide (NO), while the effect of its fraction below 3 kDa was partly explained by the SH compounds (Tavares et al., 2011).

In relation to mucus strengthening, certain food peptides have demonstrated a stimulatory activity on intestinal mucin secretion which could also contribute to their antiulcerative effect. For instance, β-casomorphin-7, a casein derived opioid peptide, induces the secretion of intestinal mucins by activation of the enteric nervous system and by a direct effect on goblet cells via interaction with μ-opioid receptors (Trompette et al., 2003; Zoghbi et al., 2006). Afterwards, this mucin stimulatory effect was also shown on goblet cells with β-lactoglobulin hydrolysed with trypsin (Martínez-Maqueda et al., 2013a) and with a peptic casein hydrolysate (Martínez-Maqueda et al., 2013b). Both hydrolysates stimulated mucin secretion and mucin 5AC gene expression in human intestinal cells HT29-MTX.

Therefore, the aim of this work was to evaluate the possible antiulcerative activity of these two hydrolysates with previously demonstrated mucin stimulatory effect. The contribution of the SH groups to the gastric protection was assessed by *in vivo* alkylation.

2. Material and methods

2.1. Production of hydrolysates

Both casein and β-lactoglobulin hydrolysates were prepared by Innaves S.A. (Porriño, Pontevedra, Spain). Hydrolysis with food-grade pepsin (Biocatalyst, Cardiff, UK) of commercial casein (Promilk 85, Arras Cedex, France) was carried out to obtain the casein hydrolysate (LowPept®), as previously described (Contreras et al., 2011). To produce the β-lactoglobulin hydrolysate, a WPC rich in β-lactoglobulin (Friesland Campina Domo, Zwolle, The Netherlands), containing at least 99.0% of β-lactoglobulin into protein content, was hydrolysed with food-grade trypsin (Biocatalyst), as reported by Martínez-Maqueda et al. (2013a).

2.2. Animals and experimental ulcerogenesis

Wistar male rats, 250-350 g body weight (bw), were obtained from the Experimental Animal Center (CEMIB) of Campinas University Campinas (Sao Paulo, Brazil). Prior experiments, animals were kept for at least seven days at 20ºC and under alternative light/dark cycles of 12 h, receiving a commercial standard diet (Nuvital Nutrients, Curitiba, Brazil) and water *ad libitum*. Animals were fasted for 24 h before experiments.

The protective effect on rat stomach mucosa was studied using the absolute ethanol ulcerogenesis model (Robert, 1979). The Ethics Committee for Animal Research of Campinas University approved the experimental protocol in agreement with the ethical principles of The International Association for the Study of Pain (IASP), regarding animal research. Animals were divided in groups of five rats, corresponding one group per treatment. Sodium chloride

solution (0.9 % w/v) at 10 mL kg^{-1} bw and an antiulcerative drug, carbenoxolone, (Sigma, St. Louis, MO, USA) at 200 mg kg^{-1} bw were used as negative and positive controls, respectively. Samples were administrated at three different concentrations (100, 300 and 1000 mg kg^{-1} bw) in a single dose by gastric intubation. One hour after sample or control ingestion, 1 mL of absolute ethanol was provided per rat. One hour later, animals were sacrificed and their stomach was extracted and washed with saline solution for ulcerative lesions analysis.

2.3. Ulcerative lesion analysis

The ulcerative lesions were evaluated by visual inspection of rat gastric mucosa. According to Gamberini et al. (1991), the ulcerative lesions index (ULI) of each animal was determined by summing the scores associated to several parameters, as follows: loss of normal morphology (1 point), mucosa discoloration (1 point), edema (1 point), hemorrhage (1 point), petechial points until 9 mm (2 points), petechial points larger than 10 mm (3 points), ulcers up to 1 mm (n x 2 points), ulcers larger than 1 mm (n x 3 points); perforated ulcers (n x 4 points), where n is the number of ulcers found.

Percent decrease of the ULI score was calculated by the expression: % decrease ULI = {(mean ULI negative control-mean ULI sample)/mean ULI negative control} x 100. The effective dose (ED50), defined as the sample concentration that achieves a decrease of 50% ULI, was estimated by using a logarithmic regression equation for ILU values obtained from 100, 300 and 1000 mg kg^{-1} treatments.

2.4. Evaluation of sulfhydryl compounds contribution

The contribution of SH in the protection of rat stomach mucosa, against the ulcerogenic activity of absolute ethanol, was evaluated by using N-ethylmaleimide (NEM). NEM has the ability to block, via alkylation, all active SH groups in the rat body (Szabo et al., 1983). The experimental protocol was analogous to that used for the study of the anti-

ulcerogenic activity of the hydrolysates, except that rats received subcutaneous injection of NEM (10 mg kg^{-1} bw) thirty minutes before the intra-gastric administration of saline solution (negative control) or hydrolysate (single concentration of 500 mg kg^{-1} bw for casein hydrolysate (CNH) and 300 mg kg^{-1} bw for WPC hydrolysate (WPH)).

2.5. Statistical Analysis

Experimental results were analysed by a one-way ANOVA, followed by Tukey's test. GraphPad Prism 4 software was used. Different letters indicate significant differences among dose groups ($P < 0.05$). * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) express statistical differences between samples and saline control.

3. Results and discussion

3.1. Evaluation of the antiulcerative properties

The antiulcerative activity of commercial casein (CN), CNH and WPH was studied in a model of ulcer induced by absolute ethanol in rats. Ethanol acts directly as an irritant agent on gastric mucosa (Robert, 1979). First, ethanol destroys the protective layer of mucosa, composed of mucus and bicarbonate, and subsequently damages the gastric epithelium, provoking necrosis, edema and hemorrhage (Oates & Hakkinen, 1988). Additionally, other ulcerogenic activities are associated to ethanol, i.e., the stimulation of stomach acid secretion, the decrease of the blood flow in the mucosa or the reduction in the PG production (Robert, 1987).

CN, CNH, and WPH were administrated at three concentrations (100, 300 and 1000 mg kg^{-1} bw) in one single dose, one hour before providing absolute ethanol. **Figure 1** shows for the different treatment groups ULI scores and ULI percent decreases compared with saline solution as control. All tested samples presented significant decreases in ULI ($P < 0.05$), compared with the saline solution, for the intermediate (300 mg kg^{-1} bw) and the highest

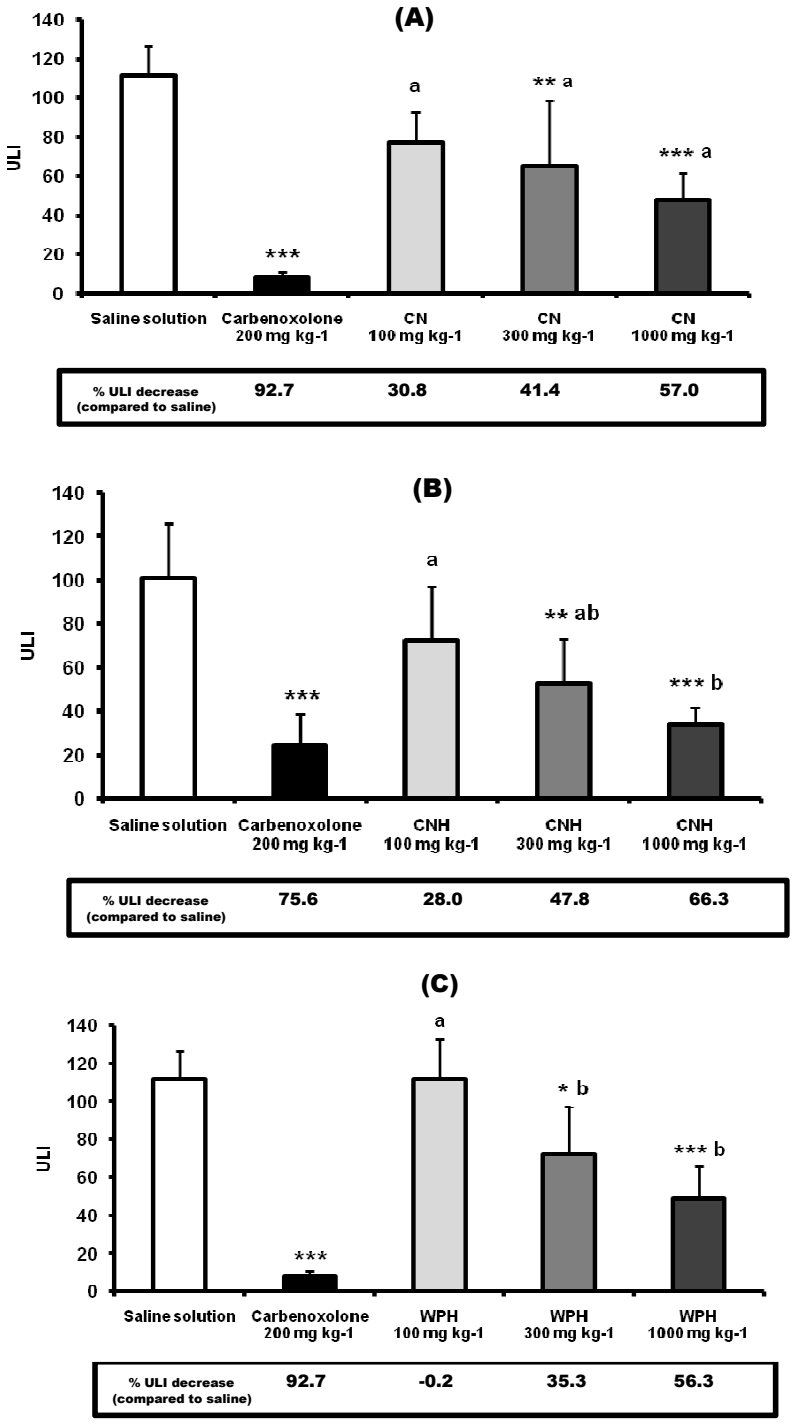

Figure 1: Effect of single dose of commercial casein (CN) (A), commercial casein hydrolysate (HCN) (B) and whey protein concentrate hydrolysate (WPH) (C) on the protective effect in rat stomachs against absolute ethanol-induced ulcers, in terms of the ulcerative lesions index (ULI), expressed as mean ± standard deviation (n=5). Data analysed by a one-way ANOVA and followed by Tukey's test. Significant differences between samples and saline solution group as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). Different letters indicate significant differences among doses ($P < 0.05$).

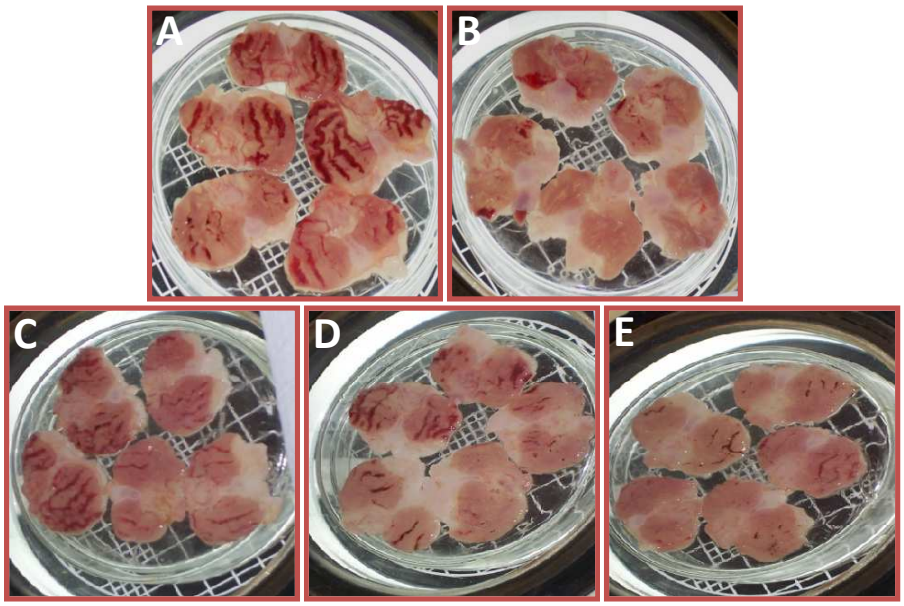

Figure 2: Images of ethanol-induced ulcers in rat stomachs under different treatment: saline solution 10 mL kg-1 body weight (bw) (A), carbenoxolone 200 mg kg-1 bw (B), commercial casein hydrolysate (CNH) 100 mg kg-1 bw (C), commercial casein hydrolysate (CNH) 300 mg kg-1 bw (D), and commercial casein hydrolysate (CNH) 1000 mg kg-1 bw (E).

concentration (1000 mg kg^{-1} bw). The effect associated to the maximum dose (1000 mg kg^{-1} bw) of all tested samples represented a notable activity against the ulcerative lesions (56.3-66.3% ULI decrease), compared with the ULI reduction registered for the antiulcerative drug, carbenoxolone, at 200 mg kg^{-1} bw (75.6-92.7%). Interestingly, a dose-response relationship was found for both hydrolysates (indicated with different letters in **Figure 1**). Although no statistical differences were appreciated between CN and CNH, the last presented an affective dose ($ED_{50)}$ of 364 mg kg^{-1}, value substantially lower than 573 mg kg^{-1} obtained for its protein source (CN) and 690 mg kg^{-1} for WPH. ED_{50}, as the dose needed to reduce the 50% percent of ulcerative lesions, was estimated from the logarithmic regression of ILU diminutions under different dose treatments.

Figure 2 shows images of the rat stomachs from different treatment groups, and the morphology of ulcers provoked by absolute ethanol and the ability of CNH to avoid these lesions on gastric mucosa. **Figure 2A** corresponds to the negative control group (saline solution treatment), where the mucosa damage can be easily appreciated as prominent red streaks. In contrast, the previous administration of carbenoxolone mostly kept the integrity of the mucosa despite irritant activity by ethanol, as shown in **Figure 2B**. **Figures 2C, 2D** and **2E** display that the higher concentration of CNH, the greater the antiulcerative effect against ethanol action. As it can be noted in **Figure 2E**, injuries were quite limited in stomachs under the protection of CNH at the highest dose, in relation to those found in the saline solution group (**Figure 2A**).

The antiulcerative activity has been described for certain food components, representing an alternative or additional therapy in the treatment of peptic ulcers. For example, food-derived polyphenols have demonstrated protective effect against lesions produced by various ulcerogenic agents and *Helicobacter pylori* (Sumbul et al, 2011; de Jesus, et al., 2012). Dairy products are considered a good source of components with antiulcerative properties, including whole and skimmed bovine milk which showed activity in

different ulcer models (Dial and Lichtenberger, 1987). The whey proteins, through WPCs, their hydrolysates and derived fractions have reported positive influence on gastric mucosa stability (Rosaneli et al, 2002; Pacheco et al., 2006; Tavares et al., 2011). Based on the research of Matsumoto et al. (2001), the protective effect exhibited by WPCs and derivates has been predominantly linked to their content in α-lactalbumin. Further studies have confirmed the ulcer inhibitory properties of this whey protein (Mezzaroba et al., 2006; Ushida et al, 2007). Matsumoto et al. (2001) found a significant inhibition of ulcer formation (47% control damage) mediated by the ingestion of a whey protein isolate (WPI), followed by the evaluation of the activity of individual components at representative concentrations, showing that only α-lactalbumin emulated the observed effect. Nevertheless, results obtained in our work for WPH differ from the established idea that β-lactoglobulin possess no significant antiulcerative properties. The commercial WPC, which was employed in the preparation of our WPH, was enriched in β-lactoglobulin up to more than 99% of the protein composition, according to previous analysis (Martínez-Maqueda et al., 2013a). Therefore, the significant activity observed for WPH with decreases of 35.3 and 56.3 % ULI, at 300 and 1000 mg kg^{-1} bw doses, respectively, are mediated by β-lactoglobulin-derived peptides, or other non-protein components. Pacheco et al. (2006) reported a comparable protective response for a pancreatic WPC hydrolysate (65.5% ULI decrease after acute 1000 mg kg^{-1} bw administration), although it must be considered that the starting WPC contained a considerable α-lactalbumin concentration (Borges et al, 2001). Anyway, results here exposed are compatible with data collected in Matsumoto et al. (2001), because only one dose of β-lactoglobulin (100 mg kg^{-1} bw) was studied showing lack of activity against alcohol-induced lesions, as occurs in the current study.

A more substantial discrepancy was found in the case of casein, since Matsumoto et al (2001) concluded that bovine casein has no antiulcerative properties after administrating 200 mg kg^{-1} bw (9% decrease ulcer index). To the best of our knowledge, no additional studies

have been published about the antiulcerative activity of casein or hydrolysates thereof. In our experiments, just the half dose of casein achieved a protective effect three times higher than the previous reported activity (30.8% ULI decrease vs 9%). These results suggest that casein may develop an important protective role versus ulcerative lesions. Such discrepancy may be related to methodological variations, such as, pre-treatment time before ethanol administration (30 min vs 60 min in our study), ethanol concentration (60% vs absolute ethanol in our study) and the ulcer analysis criteria (the injury length vs methodology from Gamberini et al (1991) in our study).

3.2. Evaluation of the contribution of sulfhydryl groups in antiulcerative activity

In order to evaluate if the observed antiulcerative activity is mediated by the action of SH groups, an *in vivo* assay using an inhibitor of SH compounds was performed. Dietary sulphur amino acids can positively influence the glutathione synthesis, which operates together some PGs with a protective role on gastric mucosa (Szabo et al., 1992; Tseng et al. 2006). The subcutaneous injection of NEM blocks this defensive route via the alkylation of all active SH groups in the rat body. The blockage of SH mechanism was assessed for both hydrolysates after acute administration (300 mg kg^{-1} bw for CNH and 500 mg kg^{-1} bw for WPH) with or without NEM preinjection. **Figure 3** shows obtained ULI scores for each treatment group, indicating also ULI reductions in relation to the reference group of each model. The alkylation of endogenous SH increased the mucosa susceptibility to ethanol-induced lesions, as can be appreciated in the rise of ULI scores from 81.2 to 137.8 due to previous injection of NEM on control saline group. The CNH antiulcerative activity was not significantly different between both models (with or without NEM), and the decrease of ULI respect each control (i.e., saline and saline+NEM) remained around 30%, indicating that its mechanism of protection was not mediated by SH. On the other hand, the inhibition of ulcer formation by WPH was drastically decreased (from 41.3 to 9.2) when rats received NEM.

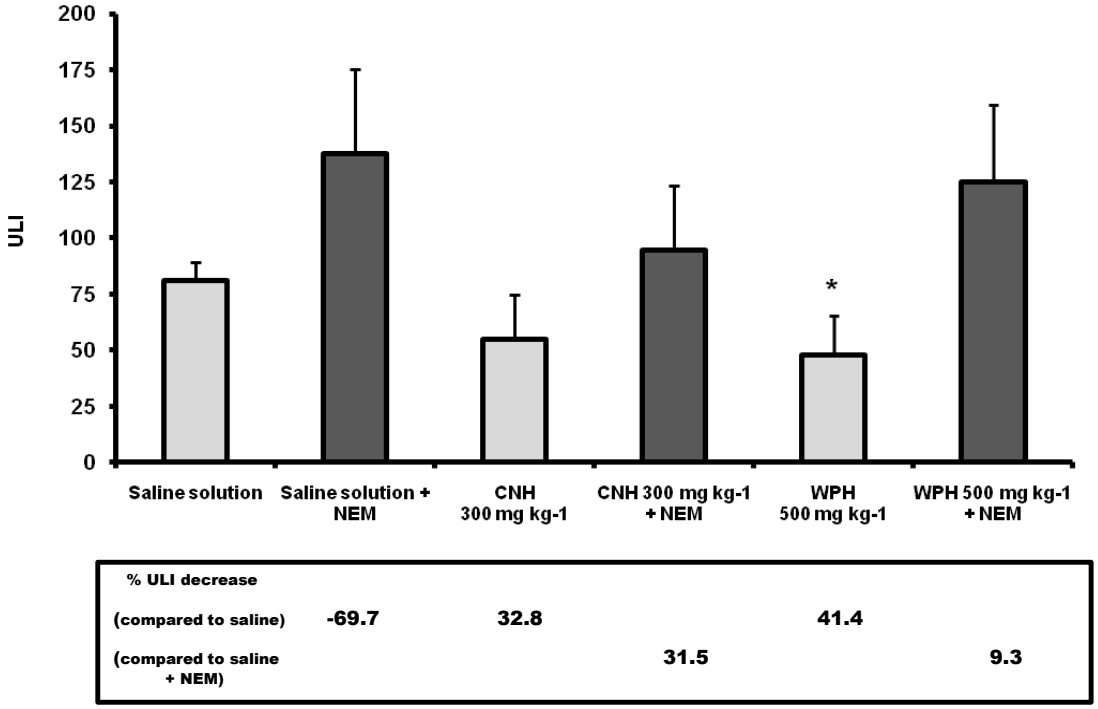

Figure 3: Effect of N-ethylmaleimide (NEM), administrated by previous subcutaneous injection at 10 mg kg^{-1} body weight, on the protective effect in rat stomachs against absolute ethanol-induced ulcers in treatment with single dose of commercial casein hydrolysate (HCN) and whey protein concentrate hydrolysate (WPH). Ulcerative lesions index (ULI) expressed as mean ± standard deviation (n=5). Data analysed by a one-way ANOVA and followed by Tukey's test. Significant differences between samples and associated saline solution group as * ($P < 0.05$).

This lack of activity under NEM injection supports the involvement of active SH in its mechanism of action on mucosa protection. **Figure 4** exposes visually the consequences of NEM pre-treatment on mucosa integrity after absolute ethanol administration. **Figure 4B, 4D** and **4F**, which correspond to rats previously treated with NEM, show stomachs clearly more damaged than those observed in **Figure 4A, 4C** and **4C**, respectively.

Loss of antiulcerative activity after *in vivo* SH alkylation might be expected in the case of WPH, in contrast to CNH, because the proportion of sulphur amino acids is considerably higher in whey proteins compared with other dietary proteins, as caseins (Smithers, 2008). Previous reports have shown that protective properties, associated to whey proteins or their hydrolysates, depend on the mechanism via active SH. For example, Rosaneli et al (2002) demonstrated an important decrease of WPC protection when endogenous SH were blocked. Mezzaroba et al. (2006) detected a similar prevented inhibition for α-lactalbumin and two α-lactalbumin-derived hydrolysate fractions on rats treated with NEM. Interestingly, two research works concluded that endogenous active SH contribute to a greater extent on antiulcerative action of low molecular weight peptides fractions compared with the corresponding whey protein-derived hydrolysates (Pacheco et al, 2006; Tavares et al., 2011). Hence, the importance of SH pathway on the gastric mucosa protection could be related to the presence of sulphur amino acids, hypothesis that explains the differences between CNH and WPH.

The stimulation of mucus production represents another important mechanism in the defence of gastric mucosa. The gastric mucus layer covers the gastric mucosa and constitutes a physical barrier between lumen and stomach tissue. Mucins, high molecular weight glycoproteins, are the main responsible for mucus viscous properties. MUC5AC and MUC6 represent the most abundant gastric secreted mucins, being MUC5AC the main component of mucus layer whereas MUC6 can develop other activities like antibiotic function against *H. pylory* (Niv and Boltin, 2012). Recently, our research group has described enhanced mucin

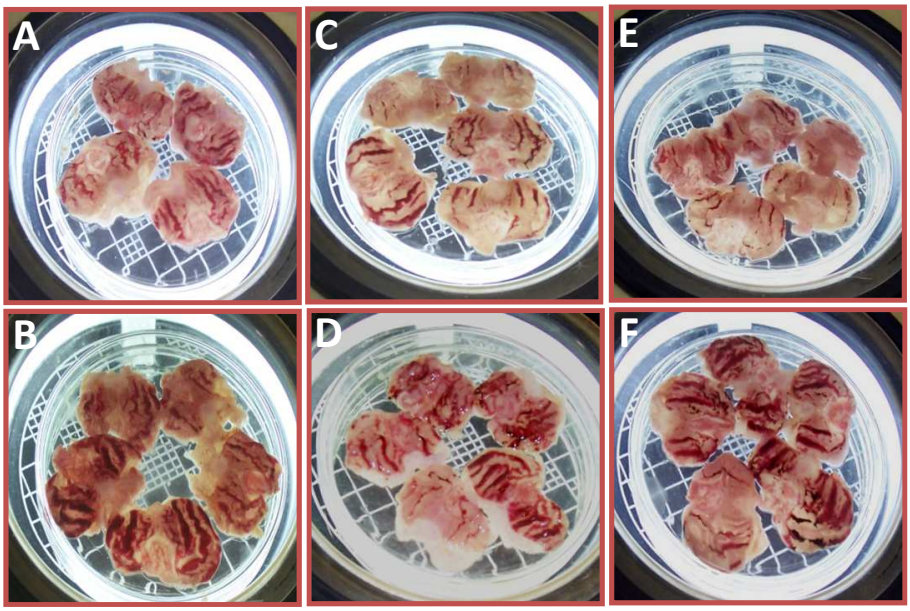

Figure 4: Images of ethanol-induced ulcers in rat stomachs under different treatment with or without previous injection of N-ethylmaleimide (NEM) at 10 mg kg^{-1} body weight: saline solution 10 mL kg^{-1} bw (A), saline solution 10 mL kg^{-1} bw + NEM (B) commercial casein hydrolysate (CNH) 300 mg kg^{-1} bw (C), CNH 300 mg kg^{-1} bw + NEM (D), whey protein concentrate hydrolysate (WPH) 500 mg kg^{-1} bw (E), and WPH 500 mg kg^{-1} bw + NEM (F).

production under the action of the hydrolysates employed in this work (CNH and WPH) when they were evaluated in intestinal human mucin-secreting HT29-MTX cells (Martínez-Maqueda et al., 2013a; 2013b). Both hydrolysates increased the mucin secretion and over-expressed the gene MUC5AC, the major secreted mucin gene in this cell line which exhibits a gastric-like mucus secreting phenotype. CNH improved the mucin secretion up to 210% of controls and up-graded the expression of MUC5AC over 1.8-fold basal level (Martínez-Maqueda et al., 2013b), demonstrating to be slightly more active than WPH, that exhibited a maximum mucin secretion of 152% of controls and a MUC5AC transcription level of 1.52-fold basal level (Martínez-Maqueda et al., 2013a). Each hydrolysate contains peptides with proved or probable μ- or δ-opioid activity whose mucin-producing activity justifies partially the detected activity of the hydrolysate. Concretely, fragments of α_{s1}-casein 90-94 (RYLGY), 143-149 (AYFYPEL) and 144-149 (YFYPEL) contained in CNH, besides β-lactorphin (YLLF) in WPH demonstrated effect on mucin secretion (Martínez-Maqueda et al., 2012; 2013a; 2013b). Therefore, it can be hypothesised that individual food-derived peptides may play a significant role in the mucosa protection via stimulation of mucus secretion. Taking into consideration the results of this work, the mucus strengthening could contribute remarkably to the antiulcerative activity, in particular for CNH whose protector effect seem to be independent from active SH groups pathway. Regardless of opioid interactions, gastric mucin release has been related to PGE_2 production (Phillipson et al., 2008). α-lactalbumin, as antiulcerative agent, initially supported this association due to the gastric mucosa protection against ulcerative ethanol or indomethacin-induced lesion via concurrent stimulation of PGE_2 and mucus increase (Mezzaroba et al, 2006), although a later *in vitro* study suggested that its mucin-producing effect was independent of endogenous PGE_2 (Ushida et al., 2007). Castro et al. (2010) demonstrated that two porcine and bovine collagen hydrolysates exerted significant gastric mucus increases, being their mucosa protective activity mostly reverted by previous NEM SH-alkylation in an absolute ethanol ulcer model. The mucus-secreting and

antiulcerative properties reported for some food-derived agents, together results obtained for CNH and WPH, agree with the mucus strengthening as key mechanism in the protective function against ethanol-induced gastric ulcers. Further studies, focusing on individual peptides with mucin-producing activity, should be carried out in rat ulcer models to identify active components in the gastric mucosa protection.

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Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin

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ABSTRACT

Dipeptidyl peptidase-IV (DPP-IV) is a serine protease involved in the degradation and inactivation of incretin hormones that act by stimulating glucose-dependent insulin secretion after meal ingestion. DPP-IV inhibitors have emerged as new and promising oral agents for the treatment of type 2 diabetes. The purpose of this study was to investigate the potential of β-lactoglobulin as natural source of DPP-IV inhibitory peptides. A whey protein concentrate rich in β-lactoglobulin was hydrolyzed with trypsin and fractionated using a chromatographic separation at semipreparative scale. Two of the six collected fractions showed notable DPP-IV inhibitory activity. These fractions were analysed by HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) to identify peptides responsible for the observed activity. The most potent fragment (IPAVF) corresponded to β-lactoglobulin f(78–82) which IC_{50} value was 44.7 μM. The results suggest that peptides derived from β-lactoglobulin would be beneficial ingredients of foods against type 2 diabetes.

1. Introduction

Diabetes mellitus is a metabolic disorder considered as one of the major health problems worldwide. It has been anticipated that the number of people affected by this chronic disease will rise to 333 million by 2025, with type 2 diabetes representing approximately 90–95% of the diagnosed cases (Pratley & Salsali, 2007). Type 2 diabetes is characterised by several pathophysiologic defects including insulin resistance, excess hepatic glucose production and progressive pancreatic β-cell dysfunction (Bharatam, Patel, Adane, Mittal, & Sundriyal, 2007). It has been estimated that 50–60% of the total insulin secreted during a meal results from the incretin response, mainly mediated by the combined effects of glucose-dependent insulinotropic (GIP) and glucagon-like peptide-1 (GLP-1) (Creutzfeldt & Nauck, 1992). GIP and GLP-1 are secreted in response to the presence of nutrients in the intestinal lumen, and act by stimulating glucose-dependent insulin secretion in the pancreatic β-cells (Holst & Deacon, 2004; Mentlein, 2005). Continuous intravenous infusion of GLP-1 normalises blood glucose concentration in diabetic subjects (Nauck et al., 1993). However, the effects of subcutaneous injections are short-lasting (Nauck et al., 1996) because of the rapid degradation and inactivation of this incretin hormone in plasma by the enzyme dipeptidyl peptidase-IV (DPP-IV). Since over 95% of GLP-1 is degraded by the action of this enzyme, specific DPP-IV inhibitors have emerged over the past few years as a new class of oral agents for the treatment of type 2 diabetes (Hunziker, Henning, & Peters, 2005; McIntosh, Demuth, Pospisilik, & Pederson, 2005). DPP-IV (EC 3.4.14.5) is a serine protease widely expressed in many tissues, including kidney, liver, lung, intestinal brush-border membranes, lymphocytes and endothelial cells. It exerts its enzymatic activity cleaving preferentially peptides with Pro or Ala residues in the second amino terminal position (Thoma et al., 2003). Many neuropeptides, cytokines, chemokines and gastrointestinal hormones, including GIP and GLP-1, are endogenous substrates for the enzyme DPP-IV which plays important roles in multiple biological processes (Cohen, Fruitier-Arnaudin, & Piot, 2004; Lambeir, Durinx, Scharpé, & De Meester, 2003). Animal experiments and human trials have demonstrated that specific DPP-IV inhibition increases the half-life of total circulating GLP-1, decreased plasma glucose, and improved impaired glucose tolerance (Deacon, Hughes, & Holst, 1998; Deacon, Nauck, Meier, Hücking, & Holst, 2000; Mitani, Takimoto, Hughes, & Kimura, 2002).

Given the high cost of treatment of type 2 diabetes-associated diseases, strategies such as dietary manipulation have been widely studied. Diet supplementation with whey protein is currently under study as a promising alternative in the prevention and/or treatment of type 2 diabetes and related-diseases in both humans and animals (Gunnarsson et al., 2006; Petersen et al., 2009). Several mechanisms of action have been suggested for whey protein, including impairing of glucose tolerance in diabetic patients, reduction of body weight, and modulation of gut hormones such

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as cholecystokinin, leptin, and GLP-1 (Sousa et al., 2012). In addition to the bioactivities exerted by the intact protein, whey proteins may exhibit further physiological functions because of bioactive peptides contained within their sequences and released during food processing or gastrointestinal digestion. Several bioactive sequences derived from whey proteins have been identified and their antihypertensive, opioid, antimicrobial, antithrombotic, mineral-binding, immunomodulant, and hypocholesterolemic properties have been reported (Hernández-Ledesma, Ramos, & Gómez-Ruiz, 2011; Hernández-Ledesma, Recio, & Amigo, 2008; Muro Urista, Álvarez Fernández, Riera Rodríguez, Arana Cuenca, & Téllez Jurado, 2011). Recently, *in silico* results reported by Lacroix and Li-Chan (2012a) have shown the promising role of whey proteins as precursors of DPP-IV inhibitory peptides. However, the potential inhibitor sequences, to become active, must be released from their parent proteins by *in vitro* hydrolysis or *in vivo* digestion. Studies carried out in the last two years have demonstrated the DPP-IV inhibitory effect of hydrolyzates of whey proteins, mainly β-lactoglobulin. However, the amount of peptides identified as potentially responsible for this effect is still scarce (Tulipano, Sibilia, Caroli, & Cocchi, 2011; Uchida, Ohshiba, & Mogami, 2011; Nongonierma & Fitzgerald, 2013).

In our laboratory, a whey protein concentrate (WPC) rich in β-lactoglobulin had been previously hydrolyzed with trypsin in optimised conditions to produce peptides with reported hypocholesterolemic, antimicrobial, opioid, and angiotensin converting enzyme inhibitory activities (Mullally, Meisel, & Fitzgerald, 1997; Nagaoka et al., 2001; Pellegrini, Dettling, Thomas, & Hunziker, 2001; Pihlanto-Leppäla, Rokka, & Korhonen, 1998). This hydrolyzate was used in the present study with the aim to evaluate its DPP-IV inhibitory activity and to identify new sequences potentially responsible for this effect.

2. Materials and methods

2.1. Materials

Bovine WPC rich in β-lactoglobulin was purchased from Friesland Foods Domo (Zwolle, The Netherlands). The protein content and pattern was analysed by SDS–PAGE and matrix assisted laser desorption ionisation-mass spectrometry (MALDI-MS). Food-grade porcine Standard Trypsin 250 USP was obtained from Biocatalysts (Nantgarw, Wales, UK). DPP-IV Drug Discovery Kit containing DPP-IV enzyme and its chromogenic substrate was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). The tripeptide IPI, called diprotin A, was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

2.2. Preparation of the whey protein concentrate hydrolyzate

The WPC (3.5 kg) was dissolved in water 5% (w/v) and heated at 90 °C for 10 min at pilot scale. The pH of the solution was adjusted at 8.0 by addition of 1 M NaOH (food grade, Aditio, Panreac Química, S.A.U., Castellar del Vallès, Spain). Trypsin was added at an enzyme-to-substrate ratio of 1:20 (w/w) and hydrolysis was carried out at 37 °C for 3 h with constant agitation. Reaction was stopped by heating at 95 °C for 15 min, assuring the complete inactivation of the enzyme. The hydrolyzate was dried by spray drying (final weight of the hydrolyzate was 3.2 kg), and the protein concentration in the final product was determined by Kjeldahl.

2.3. Assessment of the DPP-IV inhibitory activity

DPP-IV inhibitory activity was measured by a commercial *in vitro* enzymatic assay using the DPP-IV Drug Discovery Kit and following the manufacturer's instructions and the protocol described by Tulipano et al. (2011). The assay was performed in 96-well plates using diprotin A as a positive control and as a reference inhibitor. Briefly, recombinant soluble human DPP-IV (0.26 mU per test well; 15 μL) was incubated in the absence or in the presence of different concentrations of test samples (final volume 50 μL per well) at 37 °C for 10 min. The assay started after the addition of 50 μL of assay buffer containing the chromogenic substrate (H-Gly-Pro-*p*-nitroaniline) at final concentration of 100 μM. Plates were read at 405 nm in a microplate reader (BMG LABTECH Inc., Champigny sur Marne, France) at 2 min time intervals starting from 0 up to 30 min. Recorded data were plotted versus time. The best fit straight line was obtained in the time range over which the increase in OD was linear. Then, data were expressed as % remaining activity in the presence of test samples versus control (no sample added). For each sample, three different assays were performed to plot the curves that show the logarithmic regression of the dose–response curves. The results were expressed as IC_{50} value or concentration of samples needed to inhibit 50% of DPP-IV activity.

2.4. Separation of DPP-IV inhibitory peptides by semi-preparative RP-HPLC

Semi-preparative RP-HPLC was carried out according to Hernández-Ledesma, Miralles, Amigo, Ramos, and Recio (2005) with some modifications. Separation was performed on a HPLC system (Waters, Mildford, MA, USA) equipped with two pumps (module Delta 600), a pump controller (module 600), an autosampler (module 717) and a diode array detector (module 996). The data-processing software was Millenium version 32 (Waters). The WPC hydrolyzate was dissolved in Milli-Q® water at concentration of 10 mg/mL, and the injection volume was 400 μL. Fractions were eluted at a flow rate of 4 mL/min, with a linear gradient of solvent B (acetonitrile:trifluoroacetic acid (TFA) 1000:0.8 v/v) in A (water:TFA 1000:1 v/v) going from 0% to 40% B in 50 min, 40% to 70% B in 5 min, 5 min with 70% B and from 70% B to 0% B in 5 min. Each chromatographic run was repeated 15–20 times and the fractions were collected automatically with a Fraction Collector (Model II, Waters). The collected fractions were pooled, frozen and lyophilized. The peptide content of collected fractions was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) using bovine serum albumin as standard protein.

2.5. Identification of DPP-IV inhibitory peptides by RP-HPLC-MS/MS

RP-HPLC coupled to tandem mass spectrometry (RP-HPLC-MS/MS) analysis of collected fractions was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionisation source as previously described (Contreras et al., 2010). The column used was a reverse phase Mediterranea Sea C_{18} Column (150 × 2.1 mm i.d., 5 μm particle size) (Teknokroma, Barcelona, Spain). Peptides were eluted with a linear gradient of solvent B (acetonitrile:TFA 1000:0.27 v/v) in A (water:TFA 1000:0.37 v/v) going from 0% in 45% in 60 min at a flow rate of 0.2 mL/min. Using Data Analysis™ (version 3.0; Bruker Daltonics), the *m/z* spectral data were processed and transformed to representing mass values. The acquired MS/MS spectra were interpreted using BioTools (version 2.1; Bruker Daltonics).

2.6. Peptide synthesis

Synthetic peptides were prepared by the conventional Fmoc solid-phase synthesis method with a 431 A peptide synthesizer

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(Applid Biosystems Inc., Überlingen, Germany), and their purity was verified by analytical RP-HPLC-MS/MS.

2.7. Statistical analysis

Data were analysed by a one-way ANOVA, followed by the Tukey's test. GraphPad Prism 4 software was used to find significant differences between means and controls as $P < 0.001$(***), and different letters indicate significant differences among samples as $P < 0.01$.

3. Results and discussion

3.1. Fractionation of the whey protein concentrate hydrolyzate showing DPP-IV inhibitory activity

The analysis of the WPC confirmed that it contained a minimum protein content of 84% on dry matter from which 99.0% corresponded to β-lactoglobulin. In order to confirm the ability of whey proteins contained in this WPC to release DPP-IV inhibitory peptides, it was hydrolyzed with food grade trypsin for 3 h, and the DPP-IV inhibitory activity was measured before and after hydrolysis. Undigested WPC at concentration of 30 mg of protein/mL did not show inhibitory effects. However, after hydrolysis, inhibitory activity was observed and the IC_{50} value calculated for the final digest was 1.51 mg of protein/mL (final assay concentration). This result confirmed that some of the peptides released by the action of this enzyme may exert DPP-IV inhibitory activity. Lacroix and Li-Chan (2012b) reported an IC_{50} value of 0.075 mg/mL for a whey protein hydrolyzate with pepsin for 60 min. Patents WO 2006/068480 and WO 2009/128713 reported casein and lysozyme hydrolyzates prepared with different enzymes showing IC_{50} values ranged from 0.4 to 5.0 mg/mL, indicating that DPP-IV inhibiting activity is strongly influenced by the protease used to prepare the hydrolyzate (Aart, Zeeland-Wolbers, & Gilst, 2009; Pieter, 2006). In order to identify the peptides potentially responsible for the observed activity, our WPC hydrolyzate was fractionated by semi-preparative RP-HPLC. To obtain a sufficient amount of purified peptides, chromatographic separations were performed repeatedly. Six fractions (F1–F6) at 5 min intervals were collected (Fig. 1A), lyophilized, and then used to determine their DPP-IV inhibitory activity at the final concentration of 0.08 mg of protein/mL (Fig. 1B). At this concentration, fractions F5 and F6 did not show inhibitory activity, and fractions F1, and F4 inhibited 8–8.3% of the original DPP-IV activity, respectively, while fractions F2 and F3 showed higher enzyme inhibition. The IC_{50} values calculated for these two fractions were 367.3 μg/mL (F2) and 86.0 μg/mL (F3). Although fraction F3 showed higher inhibitory activity than F2, both fractions were selected to identify the peptide sequences potentially responsible for the observed activity.

3.2. Identification of potential DPP-IV inhibitory peptides

The fractions F2 and F3 were analysed by RP-HPLC-MS/MS to identify DPP-IV inhibitory peptides. As an example, Fig. 2A shows the UV-chromatogram obtained for fraction F3. The mass spectrum

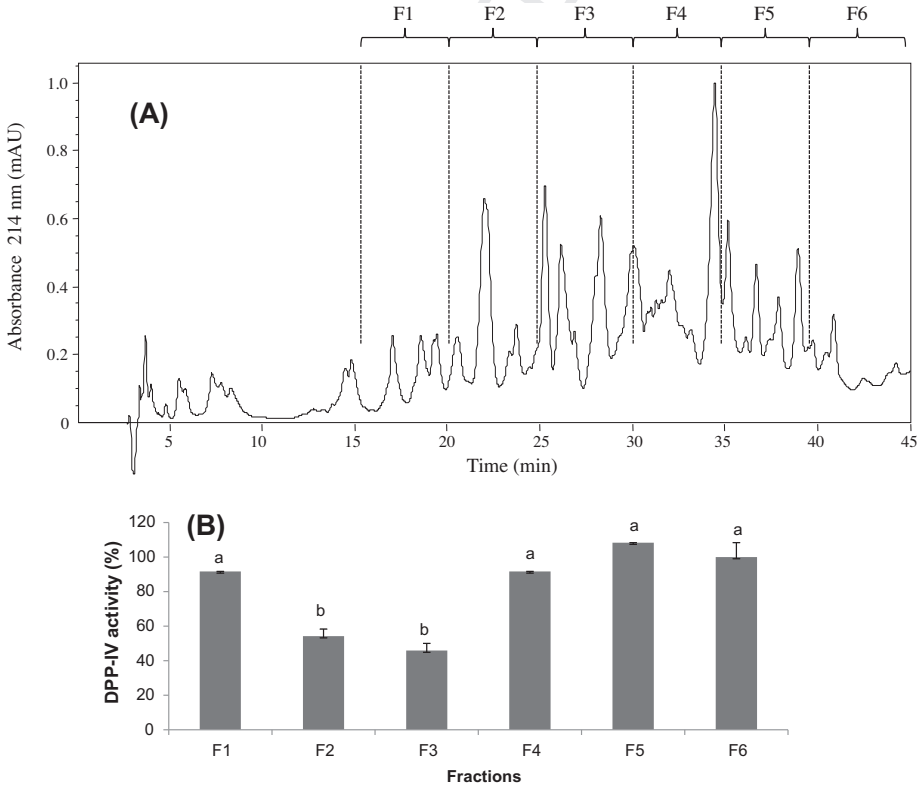

Fig. 1. (A) UV chromatogram obtained by RP-HPLC at semi-preparative scale of a whey protein concentrate rich in β-lactoglobulin ~~hydrolyzed~~ with trypsin for 3 h. Collected fractions were F1–F6. (B) Dipeptidyl peptidase-IV (DPP-IV) residual activity after incubation with collected fractions at final protein concentration of 0.08 mg/mL. Values shown are means and standard deviations (error bars), $n = 3$. Different letters indicate the significant differences among samples ($P < 0.01$) analysed by a one-way ANOVA, followed by the Tukey's test.

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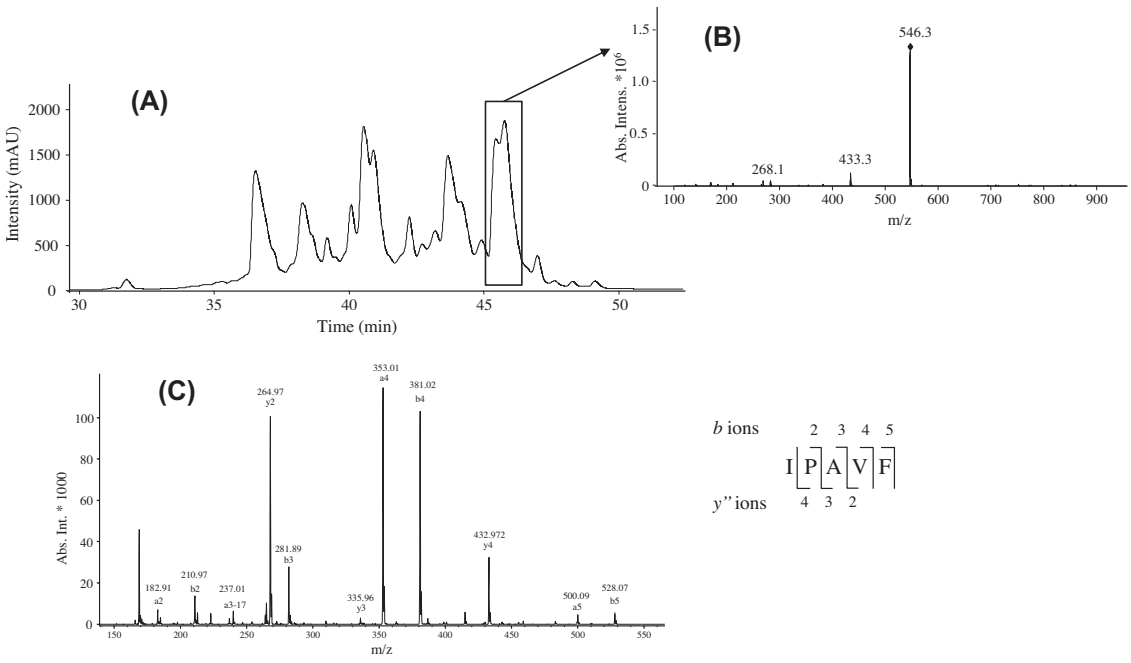

Fig. 2. (A) UV-chromatogram of the fraction F3 obtained from the whey protein concentrate rich in β-lactoglobulin hydrolyzed with trypsin for 3 h. (B) Mass spectrum of the selected chromatographic peak in (A). (C) Tandem mass spectrum of ion *m*/*z* 546.3. Following sequence interpretation and database searching, the MS/MS spectrum was matched to β-lactoglobulin f(78–82). The sequence of this peptide is displayed with the fragment ions observed in the spectrum.

Table 1
Peptides identified in collected fractions F2 and F3 from the whey protein concentrate hydrolyzate with trypsin for 3 h. The six peptides shown in bold type were synthesized to evaluate their dipeptidyl peptidase-IV (DPP-IV) inhibitory activity.

Fraction	Sequence	Fragment	IC_{50} (μM)[a] ± SD[b]
F2	**LIVTQTM**	β-Lg f(1–7)	**n.d.**[c]
	LIVTQTMK	β-Lg f(1–8)	
	VAGTWY	β-Lg f(15–20)	174.0[d]
	AGTWY	β-Lg f(16–20)	
	EILLQK	β-Lg f(55–60)	
	TPEVDDEALEK	β-Lg f(125–135)	**319.5 ± 4.0**
	LSFNPT	β-Lg f(149–154)	
F3	MAASDISLL	β-Lg f(24–32)	
	DISLL	β-Lg f(28–32)	
	TKIPAVF	β-Lg f(76–82)	
	IPAVF	β-Lg f(78–82)	**44.7 ± 3.6**
	IPAVFK	β-Lg f(78–83)	**143.0 ± 1.3**
	VLVLDTDY	β-Lg f(92–99)	
	VLVLDTDYK	β-Lg f(92–100)	**424.4 ± 31.5**
	VLVLDTDYKK	β-Lg f(92–101)	
	ALPMHIR	β-Lg f(142–148)	**n.d.**[c]

[a] The IC_{50} value was calculated as the peptide concentration required to inhibit 50% of DPP-IV activity under the assay conditions.
[b] SD: standard deviation.
[c] No inhibition was observed at higher concentration used in the study (1000 μM).
[d] Inhibitory activity measured by Uchida et al. (2011).

of selected peak and the MS/MS spectrum of ion with *m*/*z* 546.3 are shown in Fig. 2B and C, respectively. Following sequence interpretation and database searching, the MS/MS spectrum was matched to sequence IPAVF corresponding to β-lactoglobulin f(78–82). A total of 16 β-lactoglobulin-derived peptides were identified, seven of them in fraction F2 and nine in fraction F3 (Table 1). Among these peptides, the fragment f(15–20) corresponding to sequence VAGTWY, has been recently obtained after tryptic hydrolysis of β-lactoglobulin for 24 h, and described as DPP-IV inhibitor with an IC_{50} value of 174.0 μM (Uchida et al., 2011). However, to date, the inhibitory activity of the other identified peptides has not been described. Most of the inhibitory peptides reported in the literature have a length varying from 2 to 7 amino acids, and are generally characterised by their hydrophobic nature and by the presence of a Pro residue within the sequence, preferentially as first or second N-terminal residue (Huang, Jao, Ho, & Hsû, 2012). Moreover, the DPP-IV enzyme is known to cleave preferentially X-Pro and X-Ala di-peptides from the N-terminus of peptides. Many peptides displaying this characteristic have also been reported to be substrate for the enzyme. In fact, tri-peptides IPI (diprotin A) and VPL (diprotin B), both containing Pro as second amino acid residue, are substrates of DPP-IV and are commonly used as standards (Umezawa et al., 1984). Taking in account these structural features, peptides IPAVF f(78–82), IPAVFK f(78–83), and TPEVDDEALEK f(125–135), identified in our hydrolyzate, were selected to study their enzyme inhibitory activity (in bold type in Table 1). These three peptides contained Pro as second N-terminal amino acid residue, suggesting a probable DPP-IV inhibitory activity. Other peptides of F2 and F3, including LIVTQTM f(1–7), VLVLDTDYK f(92–100) and ALPMHIR f(142–148), were also selected to study their potential contribution on the activity observed in both fractions.

3.3. DPP-IV inhibitory activity of synthetic peptides

Fig. 3A and B shows the DPP-IV-inhibitory activity of the standard diprotin A (IPI), and peptide IPAVF identified in our hydrolyzate, respectively, at various concentrations. The IC_{50} values calculated for these two peptides and the rest of selected peptides are shown in Table 1. Using our spectrophotometric method to

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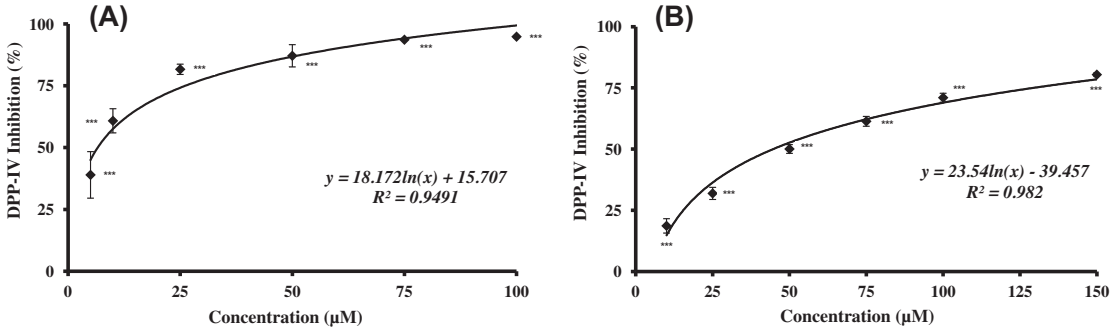

Fig. 3. Dipeptidyl peptidase-IV (DPP-IV) inhibition percentage versus peptide concentration of (A) diprotin A (peptide IPI) used as a control, and (B) peptide IPAVF identified in fraction F3 from a whey protein concentrate hydrolyzed with trypsin for 3 h. Values shown are means and standard deviations (error bars), $n = 3$. Significant differences between means and controls as $P < 0.001$(***) were analysed by a one-way ANOVA, followed by the Tukey's test.

measure DPP-IV inhibitory activity, the IC_{50} value of diprotin A was 6.6 μM. In the literature, the IC_{50} values reported for this peptide ranged from 3.5 to 24.7 μM (Huang et al., 2012; Leiting et al., 2003; Tulipano et al., 2011; Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). This variability observed among different studies may be attributable to differences in the experimental conditions, such as the type of substrate and source of enzyme used to assess the inhibitory activity.

Among the selected peptides, sequences LIVTQTM f(1–7) and ALPMHIR f(142–148), present in F2 and F3, respectively, did not show DPP-IV inhibitory activity at concentrations higher than 1000 μM. Tulipano et al. (2011) did not observe inhibitory activity when peptide ALPMH was added at concentration of 100 μM. Although presence of Leu and Pro in second and third position suggested a potential inhibitory role for these peptides, the flanking amino acids could affect the interaction between them and the enzyme (Pieter, 2006). Peptides TPEVDDEALEK and VLVLDTDYK showed moderate inhibitory activity with IC_{50} values of 319.5 and 424.4 μM, respectively. First of these peptides contains Pro as second amino acid residue, being a potential inhibitor of DPP-IV. However, the length of this fragment (eleven amino acids residues) could be the responsible for its moderate activity. As reported by Pieter (2006), best DPP-IV inhibitors are those having a length of 2–8 amino acids.

It is highlighted that among the selected peptides, sequences IPAVF and IPAVFK showed notable DPP-IV inhibitory activity. Peptide IPAVF showed an IC_{50} value of 44.7 μM (Table 1), lower than that reported by Tulipano et al. (2011) for peptide IPA. Flanking Val and Phe amino acid residues could improve the inhibitory effect. However, an additional amino acid Lys at C-terminal provoked a decrease of activity, and peptide IPAVFK showed an IC_{50} value of 143.0 μM. The presence of a Lys residue at the C-terminal end of the peptide decreases the hydrophobic character of the peptide and this could affect binding or inhibition of the enzyme. Peptide IPAVF was found as one of the major peptides in fraction F3, indicating that it could be one of the main responsible for high inhibitory activity of this fraction.

4. Conclusions

Peptides with DPP-IV inhibitory activity were released from β-lactoglobulin by hydrolysis with trypsin. Some of the identified sequences showed moderate or high inhibitory activity. It was notable the activity showed by peptide IPAVF which IC_{50} value was 44.7 μM. Further analysis should be needed to confirm *in vivo* these *in vitro* results that propose β-lactoglobulin, as source of DPP-IV inhibitory peptides, as a natural food ingredient in the prevention or co-adjuvant therapy for the management of type 2 diabetes.

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MINISTERIO DE INDUSTRIA, TURISMO Y COMERCIO

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Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

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3. DISCUSIÓN

Recientemente, ha crecido el interés por los péptidos bioactivos que pueden ejercer una acción directa en el sistema digestivo, afectando a la modulación del mismo mediante un efecto protector sobre el epitelio gastrointestinal (Shimizu & Son; 2007; Moughan et al, 2007). Gran parte de los trabajos experimentales incluidos en esta Tesis Doctoral (Publicaciones I-V) revelan nuevos datos sobre la capacidad protectora del epitelio gastrointestinal ejercida por péptidos e hidrolizados de proteínas lácteas. El fortalecimiento del mucus a través del aumento de la producción de mucinas se trata en las Publicaciones I-IV, en las cuales se describe por primera vez el efecto estimulante de ciertos péptidos alimentarios sobre la producción de mucinas por células epiteliales intestinales. Adicionalmente, se expone la actividad de dos hidrolizados (uno procedente de caseína y el otro de un WPC), que presentan incluso mayor actividad que la obtenida individualmente por los péptidos con efecto sobre la estimulación de mucinas. La Publicación IV profundiza en los mecanismos propuestos para la estimulación de la producción de mucinas, no confirmando la exclusividad de una mediación por interacciones opioides, a la vez que describe por primera vez la actividad opioide para dos secuencias de caseína. La Publicación V describe la actividad antiulcerogénica *in vivo* de los hidrolizados que previamente mostraron actividad estimulante de la producción de mucinas. Estos resultados presentan potencialidad para el desarrollo de alimentos funcionales con efecto protector sobre el epitelio gastrointestinal, principalmente frente a enfermedades gastrointestinales. Esto ha quedado recogido en la Patente I.

Adicionalmente, en esta Tesis Doctoral se ha tratado el grave problema de la diabetes mellitus y su preocupante incremento en la sociedad actual (Pratley & Salsali, 2007). El empleo de inhibidores de la enzima DPP-IV constituye una terapia alternativa para esta patología. En la Publicación VI se describe la actividad del hidrolizado de WPC rico en β-lactoglobulina como inhibidor de la enzima DPP-IV y, lo más

significativo, se describen nuevas secuencias derivadas de esta proteína de suero con una potente capacidad inhibitoria de dicha enzima.

3.1. Actividad sobre la mejora de la protección del epitelio gastrointestinal

3.1.1. Efecto estimulante de la producción de mucinas intestinales

El fortalecimiento del mucus intestinal a través del aumento de la producción de mucinas conseguido por la β-casomorfina 7 y mediado por la activación de receptores opioides, tanto en yeyuno de rata (Claustre et al., 2002; Trompette et al., 2003) como en modelos de células caliciformes intestinales humanas y de rata (Zoghbi et al, 2006), planteaba la hipótesis de si otros péptidos opioides alimentarios podrían ejercer una acción similar. La presencia de las β-casomorfinas en digeridos humanos tras la ingesta de leche (Svedberg et al., 1985) y la existencia de receptores opioides en las células caliciformes (Zoghbi et al., 2006), junto a la baja absorción de dicho péptido, al menos en individuos adultos (Read et al., 1990), hace pensar que su efecto pueda darse *in vivo* a nivel intestinal. En virtud de dichas consideraciones, se seleccionó como modelo una línea celular intestinal humana con descrita capacidad secretora de mucinas (HT29-MTX) (Lesuffleur et al., 1993). El potencial secretor de mucinas de esta línea celular fue verificado en nuestro laboratorio (Publicación I) confirmando la idoneidad de este modelo celular para el ensayo de péptidos. Los resultados obtenidos corroboraron la información existente en la bibliografía sobre la gran capacidad productora de mucinas por parte de las células caliciformes intestinales, responsables junto a los movimientos peristálticos, de la constante renovación *in vivo* del mucus intestinal (Linden et al., 2008).

Se sintetizaron distintos péptidos correspondientes a fragmentos de proteínas alimentarias, con actividad opioide tipo μ o δ descrita o cuyas secuencias presentaran una estructura favorable para la interacción con receptores opioides, presentando un

residuo de tirosina en la primera o segunda posición amino-terminal y otro aminoácido aromático (tirosina o fenilalanina) en la tercera o cuarta posición (Meisel & FitzGerald, 2000). En un primer estudio (Publicación I) se determinó la actividad estimulante sobre la secreción de mucinas en el modelo celular HT29-MTX. Además de confirmar la actividad secretora de la β-casomorfina 7, previamente descrita, y el acusado efecto de una encefalina μ-específica (DAMGO), seis de las ocho secuencias peptídicas evaluadas presentaron incrementos estadísticamente significativos de la secreción de mucinas, oscilando su valor máximo entre 157 y 453% respecto al control (células no tratadas). Entre los péptidos ensayados, la exorfina A5 procedente de la glutenina de trigo, presentó el menor incremento pero representa el primer caso descrito de actividad secretora de mucinas en células caliciformes por parte de un péptido de procedencia vegetal. El efecto estimulante sobre la expresión de MUC5AC, el gen de la mucina secretada más abundante en esta línea celular, fue estudiado para la α-lactorfina y la β-casomorfina 5 humana, dos de los péptidos opioides que habían mostrado valores destacados de actividad estimulante sobre la secreción de mucinas. A pesar de mostrar efectos estimulantes similares sobre la secreción de mucinas, la respuesta en la expresión de MUC5AC fue distinta, presentando la α-lactorfina una tendencia incrementada entre las 4 y 24 h, con un aumento significativo a las 24 h, mientras que la β-casomorfina 5 no presentó sobreexpresión significativa a los tiempos estudiados (Publicación I). Aunque previamente se había descrito la actividad productora de mucinas por parte de la α-lactoalbúmina en una línea celular de mucosa gástrica de rata (Ushida et al., 2007) y de un hidrolizado de esta proteína en yeyuno de rata (Claustre et al., 2002), los resultados de nuestro estudio permitieron caracterizar la actividad estimulante sobre la secreción y expresión de mucinas en células caliciformes intestinales, de una secuencia (α-lactorfina) derivada de dicha proteína de suero.

En un segundo trabajo (Publicación II) se propuso estudiar el efecto estimulante que la β-lactorfina (forma amidada), un péptido opioide derivado de la β-lactoglobulina bovina, tiene sobre la expresión de MUC5AC. Este péptido había sido identificado (Publicación I) como la secuencia que mayor secreción de mucinas provocó en la evaluación inicial (453% del control). Se pudo comprobar un claro y significativo efecto estimulante sobre la expresión de MUC5AC, alcanzando hasta 2,2 veces la expresión basal de dicho gen. La forma amidada de la β-lactorfina no se presenta de manera natural en la naturaleza, por lo que se procedió a estudiar la actividad de la forma no amidada, en la que también se había descrito cierta actividad opioide (Antila et al., 1991). Los resultados mostraron un incremento estadísticamente significativo sobre la secreción de mucinas a la concentración de 0,5 mM de un 152% del control pero que no se vio reflejado en ninguna sobreexpresión de MUC5AC en los tiempos estudiados.

En un posterior trabajo (Publicación III) se planteó la evaluación a nivel transcripcional de la actividad de dos secuencias derivadas de la α_{s1}-caseína bovina, en concreto los fragmentos 90-94 (RYLGY) y 143-149 (AYFYPEL) que previamente mostraron influencia positiva sobre la secreción de mucinas (Publicación I), a pesar de no presentar actividad opioide descrita aunque sí una estructura favorable para ello. Dichos péptidos son responsables de la actividad antihipertensiva de un hidrolizado de caseínas (Contreras et al., 2009), en el que se encuentran en concentraciones similares a otras secuencias que presentan potencialidad de interaccionar con receptores opioides, los fragmentos 144-149 de la α_{s1}-caseína (YFYPEL) y 89-95 de la α_{s2}-caseína (YQKFPQY). Aunque RYLGY mostró niveles de ARNm algo superiores a los controles, sólo AYFYPEL e YFYPEL provocaron un aumento significativo de la expresión de MUC5AC, logrando incrementos similares y cercanos a 1,8 veces la expresión basal. Para estas dos secuencias se determinó su efecto sobre la actividad secretora de mucinas, volviendo a mostrar incrementos significativos máximos similares, siendo algo superiores a 160% del control.

Con el objetivo de estudiar el fragmento mínimo activo sobre la producción de mucinas dentro de la secuencia 143-149 de la α_{s1}-caseína (AYFYPEL) y una posible relación estructura-actividad, se planteó determinar la expresión de MUC5AC bajo la acción de dos secuencias derivadas, YFYPE e YFYP, que siguen presentando una estructura favorable para interaccionar con receptores opioides (Publicación IV). Tras el tratamiento con dichos péptidos a las concentraciones de 0,05, 0,1 y 0,5 mM, no se encontró ningún incremento significativo frente al control, por lo que se concluyó que YFYPEL constituye el fragmento mínimo con actividad estimulante sobre la producción de mucinas en dicha secuencia estudiada. Paralelamente, se decidió estudiar la actividad opioide de los péptidos comprendidos en la citada secuencia 143-149 de la α_{s1}-caseína, incluyendo AYFYPEL, YFYPEL, YFYPE e YFYP. Para ello se empleó el ensayo en músculo longitudinal-plexo mientérico de íleon de cobayo, en el que la inhibición de las contracciones inducidas eléctricamente refleja interacciones con los receptores opioides tipo μ y κ localizados (Schwartz et al., 1997). El efecto se verificó además mediante la reversión con un antagonista opioide (naloxona). Como control positivo se tomó β-casomorfina 7, que registró un 20,1% de inhibición máxima, siendo superada en actividad por YFYPEL e YFYPE que obtuvieron 25,5 y 30,9% de actividad máxima inhibitoria, respectivamente, y se describieron por primera vez como secuencias opioides. Por otro lado, AYFYPEL e YFYP no presentaron actividad significativa. Es de destacar que al analizar la estabilidad de YFYPEL e YFYPE en el baño de órganos, se comprobó que la concentración de dichos péptidos disminuía drásticamente al cabo de unos minutos, probablemente por la actuación de peptidasas existentes en el tejido de íleon de cobayo. Esta situación puede ser la responsable de la falta de una respuesta dosis-actividad para estas secuencias. El análisis de los datos plantea una aparente falta de correspondencia entre la actividad opioide, medida en íleon de cobayo, y el efecto estimulante sobre la expresión de mucinas en las células caliciformes. Aunque AYFYPEL no demuestra actividad opioide destacable, da

lugar a una sobreexpresión similar del gen MUC5AC a la obtenida por YFYPEL, que sí exhibe una actividad opioide significativa. El péptido YFYPE presenta una respuesta opioide similar a la obtenida para el péptido YFYPEL, pero no presenta incremento significativo sobre la expresión de mucinas.

Con el fin de profundizar en el mecanismo implicado en la estimulación de la producción de mucinas por parte de los péptidos identificados, se procedió a examinar la variación de la actividad del péptido YFYPEL durante el tratamiento conjunto con ciprodima, un antagonista μ-opioide (Publicación IV). Ni individualmente frente al control, ni en conjunto con YFYPEL frente a la respuesta individual del péptido, la presencia del antagonista provocó cambio significativo alguno en los niveles de transcripción de MUC5AC. Esto podría interpretarse descartando el mecanismo mediado por receptores opioides tipo μ, al menos en el caso de este péptido. Este mecanismo fue el inicialmente propuesto y demostrado para el estímulo de la secreción de mucinas por parte de la β-casomorfina 7 y un hidrolizado de caseínas comercial en un modelo de yeyuno de rata (Claustre et al., 2002). Posteriormente se comprobó en cultivos celulares de células caliciformes, que la actividad estimulante de la β-casomorfina 7 sobre la secreción y expresión de mucinas se producía a través de interacciones opioides tipo μ, en base a la reversión del efecto tras el bloqueo de los receptores opioides tipo μ con ciprodima (Zoghbi et al., 2006). Por el contrario, un trabajo reciente de Plaisancié et al. (2013) describe la actividad estimulante sobre la secreción de mucinas y la expresión de MUC2 y MUC4 en HT29-MTX de la secuencia 94-123 (GVSKVKEAMAPKHKEMPFPKYPVEPFTESQ) de la β-caseína, encontrada en el conjunto de péptidos de un yogurt, donde se verificó la no reversión de la actividad por tratamiento previo de las células con el antagonista ciprodima. Respecto a los resultados de actividad estimulante de la producción de mucinas recogidos en nuestros ensayos (Publicaciones I-IV), es interesante valorar las actividades observadas en relación a su mayor o menor selectividad frente a receptores opioides.

En el primer trabajo es de destacar que la α-lactorfina consiguió sobreexpresar el gen MUC5AC, a diferencia de la β-casomorfina 5 humana que no mostró efecto estimulante alguno sobre la expresión de dicho gen, aunque ambos demostraran resultados similares sobre la secreción de mucinas (Publicación I). Esta actividad está en discrepancia con la interacción opioide mostrada por ambas secuencias en experimentos con íleon de cobayo, donde la β-casomorfina 5 humana poseía unas 2,6 veces menos actividad opioide que la β-casomorfina 7 bovina, mientras que la α-lactorfina unas 7,2 veces menos, con lo que claramente la β-casomorfina 5 humana se presenta con mayor actividad opioide que la α-lactorfina (Koch et al., 1985; Yoshikawa et al., 1986). Por otro lado, la exorfina A5 de glutenina de trigo consiguió la menor estimulación secretora de mucinas, resultado que podría discutirse desde la mayor afinidad descrita frente a receptores tipo δ en comparación con la mostrada frente a receptores tipo μ (Teschemacher, 2003). La influencia de la actividad opioide sobre el efecto estimulante en la producción de mucinas está bien caracterizada en el caso de la β-lactorfina (Publicación II), donde la forma amidada obtuvo un mayor efecto estimulante tanto en la secreción como en la expresión de mucinas frente al obtenido por la forma no amidada. La afinidad opioide para la forma amidada ha sido descrita como superior a la correspondiente de la forma no amidada, lo que podría explicar estos resultados (Yoshikawa et al., 1986; Antila et al., 1991). El análisis de todos los datos obtenidos en estos trabajos sobre el estímulo de la secreción y la expresión de mucinas en cultivos de células caliciformes intestinales (Publicaciones I-IV), no permite evidenciar que el mecanismo esté exclusivamente mediado por interacciones con receptores opioides tipo μ, tal como se describió en el caso de la β-casomorfina 7, aunque en algunos casos si parece tener participación. Se deberían llevar a cabo ensayos de inhibición adicionales con otras secuencias activas, empleando distintos antagonistas opioides, como la naloxona que interacciona con todos los receptores opioides. El procedimiento experimental por adición simultánea, tal como se ha llevado

a cabo en la Publicación IV, o el pretratamiento con el antagonista, tal como hizo el grupo de investigación de la Dra. Plaisancié, podría ser otro factor a tener en cuenta.

Aunque se ha descrito que la actividad enzimática de la línea celular parental HT29 es menor a la observada en otras líneas celulares intestinales, como la Caco-2 (Howell et al., 1992), se planteó la evaluación de la estabilidad de los péptidos empleados. Las peptidasas presentes en la línea celular podrían degradar los péptidos, liberándose formas inactivas o bien generándose nuevas formas activas estimulantes de la producción de mucinas. El análisis de los sobrenadantes de las células tratadas con la β-lactorfina en su forma no amidada, mostró una estabilidad mantenida durante las primeras horas de tratamiento, presentando a las 4h una señal de la corriente total de iones en espectrometría de masas de aproximadamente el 85% de la inicial. A las 24 h la señal se vio sensiblemente disminuida pero no se identificó ningún fragmento derivado, por lo que podría estar produciéndose una inclusión del péptido en la capa mucosa o en el interior celular (Publicación II). También se determinó la estabilidad de los péptidos RYLGY, AYFYPEL, YFYPEL e YQKFPQY a lo largo de las 24 h de tratamiento (Publicación III) y no se observó degradación apreciable durante las primeras 8h. La acción de las peptidasas sobre AYFYPEL generó el fragmento YFYPEL, activo como estimulante de la producción de mucinas, aunque en una proporción tan pequeña que no permite responsabilizarlo de la actividad observada para AYFYPEL a las 4h. Los resultados de estabilidad observados para distintas secuencias y los tiempos registrados para la mayoría de los incrementos significativos, sugieren que la degradación de péptidos por parte de las peptidasas celulares no es la causa de la falta de actividad en aquellos péptidos que no han mostrado efecto aparente sobre la estimulación de la secreción o expresión de mucinas.

Una vez conocida la actividad de péptidos concretos, el siguiente paso fue la preparación y evaluación en células HT29-MTX de la actividad de hidrolizados que los

contuvieran. La β-lactorfina en su forma no amidada se obtuvo e identificó tras la hidrólisis con tripsina de grado alimentario de un WPC enriquecido en β-lactoglobulina. Más de un 99% del contenido proteico del WPC corresponde a β-lactoglobulina, tal como se determinó por electroforesis en gel de poliacrilamida (SDS-PAGE) y por espectrometría de masas MALDI-TOF (Publicación II). Dicho hidrolizado demostró actividad estimulante tanto sobre la secreción como sobre la expresión de mucinas, alcanzando un incremento significativo de 152% respecto a la secreción de mucinas por parte del control y una sobreexpresión de 1,5 veces la basal del gen MUC5AC. En la caracterización del hidrolizado por HPLC-MS/MS, entre los más de 40 péptidos identificados, la β-lactorfina fue el único péptido con una estructura que permitiera anticipar una posible interacción con los receptores opioides. La capacidad estimulante sobre la secreción de mucinas por parte del hidrolizado se podría justificar por la actividad detectada para la β-lactorfina en su forma no amidada pero, sin embargo, dicha β-lactorfina no mostró efecto sobre la expresión de MUC5AC como sí lo hizo el hidrolizado.

Se estudió un hidrolizado comercial de caseínas, cuya actividad antihipertensiva se atribuye principalmente a los fragmentos de la α_{s1}-caseína RYLGY y AYFYPEL (Contreras et al., 2009; 2011). Además de dichos péptidos, el hidrolizado contiene en concentraciones similares los péptidos YFYPEL e YQKFPQY, cuya estructura sugiere una potencial interacción con receptores opioides. Al ensayar la actividad estimulante sobre la producción de mucinas, el hidrolizado de caseína demostró efecto sobre la expresión de MUC5AC, alcanzando un máximo estadísticamente significativo de 1,8 veces la expresión basal, a la vez que incrementó la secreción de mucinas hasta un 210% del obtenido para el control (Publicación III). Los péptidos activos AYFYPEL e YFYPEL, a pesar de encontrarse en el hidrolizado a una concentración algo inferior a la ensayada individualmente, podrían justificar la sobreexpresión observada en base a sus actividades individuales. No obstante, el hidrolizado de caseínas mostró un efecto

estimulante sobre la secreción de mucinas de mayor intensidad y en un momento posterior del tratamiento (8 y 24h vs 2 y 4 h) al mostrado por los péptidos AYFYPEL e YFYPEL. Tanto para el hidrolizado de caseínas comercial, como para el hidrolizado de WPC (principalmente β-lactoglobulina), se describen destacables efectos inductores de la producción de mucinas. La actividad mostrada por los hidrolizados no puede explicarse exclusivamente en base al efecto individual mostrado por los péptidos con actividad opioide o con una estructura que pudiera interaccionar potencialmente con los receptores opioides. Estos resultados llevan a la conclusión de que otros péptidos o componentes podrían actuar de forma sinérgica o a través de un mecanismo distinto a la activación de receptores opioides. En esta línea, Sprong et al. (2010) observó un aumento en las mucinas fecales tras la ingesta de un suero de quesería por parte de ratas a las que se había inducido inflamación intestinal mediante dextrán sulfato sódico. El efecto conseguido fue discutido bajo la hipótesis del contenido de treonina y cisteína en el suero de quesería, al ser estos aminoácidos limitantes en la síntesis de mucinas bajo esas condiciones patológicas (Faure et al., 2006). La hipótesis estaba respaldada en que no se observaba ninguna sobreexpresión de Muc2, el gen de la principal mucina secretada, y que la actividad obtenida por caseína suplementada en dichos aminoácidos era similar a la mostrada por el suero de quesería, a la vez que distinta a la caseína sin suplementar. Este mecanismo podría aplicarse parcialmente al hidrolizado de proteínas de suero, en base a su equiparable contenido en cisteína y treonina, aunque hay que tener en cuenta que en nuestro caso el hidrolizado sí consigue cambios a nivel transcripcional, sobreexpresando hasta 1,8 veces el gen MUC5AC.

Los tiempos de tratamiento, en los que se presentan con más frecuencia los fenómenos de estimulación de la producción de las mucinas, corresponden preferentemente a tiempos cortos, como 2 y 4 h. Es relevante destacar que, a excepción de la α-lactorfina, la totalidad de péptidos e hidrolizados que han provocado

sobreexpresión del gen MUC5AC en las células HT29-MTX lo han hecho a las 4h. Un trabajo reciente (Plaisancié et al., 2013) realiza todas sus medidas de actividad sobre la producción de mucinas en HT29-MTX directamente a las 4h, lo que podría deberse a la selección de dicho tiempo en base a medidas previas. En trabajos anteriores, la β-casomorfina 7 bovina había mostrado actividad estimulante sobre la secreción de mucinas a las 4 y 8 h, aunque la sobreexpresión del gen MUC5AC se detectó a las 24h (Zoghbi et al, 2006). De la misma forma, se observó incremento en la expresión génica de mucinas para un compuesto no peptídico, el ácido graso de cadena corta butirato, sólo tras 24 h y no a los tiempos inferiores de 1, 3 y 8 h de tratamiento en una línea celular derivada de HT29 (Gaudier et al., 2004). Posteriormente, se volvió a ver actividad estimulante de ácidos grasos de cadena corta sobre la expresión de MUC2 en un cultivo de células caliciformes humanas (LS174T) a las 24 h, aunque no se estudió a tiempos más cortos (Burger-van Paassen et al., 2009). Concretamente, la α-lactorfina demostró un aumento significativo de los niveles de ARNm del gen MUC5AC a las 24 h, aunque presentó valores incrementados a tiempos menores, que no llegaban a alcanzar validez estadística por la variabilidad biológica existente (Publicación I). La secreción de mucinas seguida de un aumento en la expresión de los genes responsables, se asocia con un fenómeno de recarga de la reserva intracelular de mucinas, comportamiento observado en otros tipos de células secretoras como las células β pancreáticas responsables de la síntesis de insulina (Webb et al., 2000). Este patrón se ha podido advertir en los casos del hidrolizado de WPC, donde la secreción se incrementó a las 2 h mientras que la sobreexpresión de MUC5AC se dio a las 4h (Publicación II), y parcialmente en el tratamiento con YFYPEL donde la secreción se presentó a las 2 y 4 h mientras que la expresión exclusivamente a las 4h (Publicación III). El caso totalmente contrario se puede apreciar en la acción del hidrolizado de caseínas, el cual produjo incrementos bastante destacables a las 8 y 24 h, tiempos posteriores a la sobreexpresión ocurrida a las 4h (Publicación III). También existen ejemplos de concurrencia de efectos como son la β-lactorfina

amidada y la secuencia caseínica AYFYPEL. En base a los datos recogidos, no parece fácil pronosticar un comportamiento concreto sobre la inducción de mucinas en las células HT29-MTX, aunque parece que lo más previsible podría ser la sobreexpresión de mucinas a las 4h y la estimulación de la secreción de mucinas en los primeros tiempos (2 y/o 4h). La actividad estimulante de mucinas *in vitro* a tiempos cortos podría tener una importante relevancia *in vivo* a nivel fisiológico. De hecho, resultados preliminares en animales han demostrado sobreexpresión de los genes Muc2 y Muc4 en yeyuno e íleon bajo la acción del hidrolizado de WPC (resultados no mostrados).

3.1.2. Actividad antiulcerogénica de hidrolizados proteicos

Se propuso la evaluación de la actividad antiulcerogénica para los dos hidrolizados que previamente habían mostrado efecto estimulante sobre la producción de mucinas en células caliciformes intestinales, específicamente, el hidrolizado del WPC enriquecido en β-lactoglobulina (Publicación II) y el hidrolizado comercial de caseína (Publicación III). La estimulación de la producción de mucus representa, también en la mucosa gástrica, un importante mecanismo de intervención en la protección del epitelio (Niv & Boltin, 2012). MUC5AC representa la mucina secretada más abundante en el estómago, siendo también la principal mucina producida en la línea celular intestinal HT29-MTX, ya que presenta un fenotipo mucosecretor gástrico como resultado de su adaptación al metotrexato (MTX) (Lesuffleur et al., 1993). Para el estudio se empleó un modelo *in vivo* de ulcerogénesis inducida por etanol absoluto en rata, mediante la administración directa de 1 mL a cada animal. El etanol actúa como un agente irritante en la mucosa gástrica, ya que inicialmente destruye la capa de mucus y bicarbonato, dañando a continuación el epitelio y provocando necrosis, edema y hemorragia (Robert, 1979; Oates & Hakkinen, 1998). Adicionalmente a los hidrolizados, también se estudió la actividad antiulcerogénica de la caseína comercial de partida.

La administración intragástrica de la caseína, el hidrolizado de caseína y el hidrolizado de WPC se llevó a cabo a tres dosis (100, 300 y 1000 mg kg^{-1} de rata), mostrándose inhibiciones significativas del daño gástrico para las tres muestras a las dosis intermedia y superior (Publicación V). Los valores medios de inhibición más altos se obtuvieron para el hidrolizado de caseínas, alcanzando hasta un 66.3% de protección frente al 75.6% generado en el mismo experimento por el fármaco carbenoxolona. Aunque no se observaron diferencias significativas entre el hidrolizado de caseínas y la caseína de partida, sí se advirtió dosis-respuesta para el hidrolizado, al igual que en el de WPC. Los resultados alcanzados en este estudio tienen una relevancia considerable ya que son los primeros que asocian actividad antiulcerogénica a un hidrolizado procedente casi exclusivamente de β-lactoglobulina, además de a la caseína y un hidrolizado derivado de ella. Otros concentrados de proteínas de suero y sus hidrolizados han mostrado actividad protectora frente a úlceras gástricas inducidas por etanol u otros agentes (Rosaneli et al., 2002; Pacheco et al., 2006; Tavares et al., 2011), aunque dicha actividad ha sido atribuida a su contenido en α-lactoalbúmina. Efectivamente, el efecto protector en la mucosa gástrica de la α-lactoalbúmina ha sido ampliamente verificado (Ushida et al., 2003; 2007; Mezzaroba et al., 2006). La premisa de considerar a la α-lactoalbúmina como principal responsable de la actividad antiulcerogénica se basa en un estudio de Matsumoto et al. (2001), que describe la inhibición del desarrollo de úlceras en la mucosa gástrica tras aplicar un aislado de proteínas de suero. En dicho estudio se ensayaron posteriormente, por separado, las distintas proteínas del aislado (entre ellas la β-lactoglobulina) a la dosis correspondiente con su concentración en el aislado, encontrándose que sólo la α-lactoalbúmina era capaz de emular el efecto conseguido por el aislado. El papel antiulcerogénico de la β-lactoglobulina y, en concreto, de los péptidos derivados de ella, toma relevancia en base a los resultados obtenidos para el hidrolizado de WPC enriquecido en dicha proteína sérica, dónde esta proteína representaba más del 99% del contenido proteico del concentrado (Publicación V). No

se puede excluir que otros componentes no proteicos del hidrolizado puedan participar en dicha actividad. La falta de capacidad protectora de la β-lactoglobulina, descrita en el trabajo de Matsumoto et al., estaría en línea con la ausencia de inhibición de lesiones ulcerativas recogida en nuestro estudio para su hidrolizado a la misma dosis empleada (100 mg kg^{-1}). No obstante, hay que destacar que a partir de 300 mg kg^{-1} de hidrolizado ya muestra una inhibición estadísticamente significativa. De hecho, la protección observada bajo este hidrolizado es comparable con la obtenida para un hidrolizado con pancreatina de un concentrado de proteínas de suero con mayor proporción de α-lactoalbúmina (Borges et al., 2001), alcanzando un 65,5% de inhibición a la dosis de 1000 mg kg^{-1} (Pacheco et al., 2006).

En el caso de las caseínas, igualmente Matsumoto et al. (2001) estudiaron su efecto *in vivo* en un modelo de úlcera gástrica inducida por etanol, llegando a la conclusión de que no presentaba actividad tras ensayarla a 200 mg kg^{-1} y registrar tan sólo una disminución del 9% en el daño gástrico. Ese resultado hizo considerar a la caseína bovina como no activa en la protección del epitelio gástrico, no llevándose a cabo ningún estudio posterior relacionado. Aunque no llegó a representar una disminución significativa frente al control salino en nuestro experimento, la mitad de dicha dosis de caseína (100 mg kg^{-1}) generó una reducción media del más del triple (30,8 vs 9% reducción en ULI), alcanzando significancia estadística bajo la dosis de 300 mg kg^{-1} con un 41.4% de reducción (Publicación V). Dicha discrepancia de resultados puede estar basada en diferencias metodológicas entre ambos estudios, como son el distinto tiempo de actuación de la caseína (30 min vs 60 min en el nuestro), la concentración del agente ulcerogénico (60% etanol vs etanol absoluto en el nuestro) y el criterio para el análisis de las úlceras (longitud de las mismas vs la sistemática de Gamberini et al. (1991) en el nuestro). En base a los resultados recogidos, tanto la caseína comercial como un hidrolizado comercial de la misma con pepsina, tienen comprobada actividad protectora de la mucosa gástrica frente a

úlceras inducidas por etanol absoluto (Publicación V). Aunque el hidrolizado obtiene valores medios de inhibición algo superiores que la caseína completa, la diferencia entre los grupos no llega a ser estadísticamente significativa. Teniendo en cuenta que el hidrolizado de caseínas se preparó con pepsina, los péptidos contenidos en el hidrolizado deben ser análogos a los generados *in vivo* por la acción de la pepsina gástrica. No obstante, el grado de hidrólisis es mayor en el hidrolizado por el mayor tiempo de actuación de la pepsina, hecho que apoyaría los valores de inhibición ligeramente superiores del hidrolizado frente a la propia caseína completa, aunque sin significancia estadística.

Con el objetivo de profundizar en el mecanismo implicado en la actividad antiulcerogénica demostrada por los dos hidrolizados estudiados (caseína y WPC), se planteó un estudio sobre la participación de los grupos SH activos. La inyección de N-etilmaleimida (NEM) provoca la alquilación *in vivo* de grupos SH, bloqueando dichos compuestos y permitiendo valorar la contribución de dicha ruta de defensa. El estudio sobre una dosis de cada uno de los hidrolizados (300 mg kg^{-1} para el de caseínas y 500 mg kg^{-1} para el de WPC), mostró que la actividad del hidrolizado de WPC se veía significativamente disminuida (pasando de 41,4 a 9,3% de reducción), mientras que la respuesta del hidrolizado de caseína no se veía apenas modificada (Publicación V). Las propiedades antiulcerogénicas mediadas por el mecanismo de grupos SH suelen provenir principalmente por la acción de los aminoácidos azufrados, los cuales favorecen la síntesis del glutatión, que a su misma vez actúa junto a algunas prostaglandinas con un papel protector del epitelio gástrico (Szabo et al., 1992; Tseng et al., 2006). Por ello, la circunstancia de que la actividad protectora mostrada por el hidrolizado de WPC se vea afectada por la alquilación de grupos SH, mientras que el efecto del hidrolizado de caseína, no, está en consonancia con el destacado contenido de estos aminoácidos en las proteínas de suero respecto al de las caseínas (Smithers, 2008). Existen varios casos descritos de pérdida de actividad antiulcerogénica en

proteínas de suero o hidrolizados de las mismas, al bloquear el mecanismo de grupos SH mediante la preinyección con NEM. Rosaneli et al. (2002) observaron una importante disminución de actividad en un WPC tras la alquilación *in vivo* de grupos SH, de forma similar a lo apreciado por Mezzaroba et al. (2006) para la propia α-lactoalbúmina y dos fracciones de hidrolizados derivados. Este mecanismo parece tener mayor repercusión en la actividad antiulcerogénica de las fracciones de menor peso molecular que en aquella de los hidrolizados completos (Pacheco et al., 2006; Tavares et al., 2011).

La capacidad productora de mucinas, mostrada en células caliciformes intestinales por parte de los hidrolizados de caseína y WPC (Publicaciones II y III), podría desempeñar un papel crucial en el efecto protector evidenciado por ellos en el modelo de úlcera inducida por etanol (Publicación V). Castro et al. (2010) describió que dos hidrolizados de colágeno (bovino y porcino) aumentaban la secreción de mucus gástrico, a la vez que su efecto antiulcerogénico desaparecía mayoritariamente previa alquilación de los grupos SH con NEM. Un comportamiento similar fue observado para la α-lactoalbúmina, actuando con incrementos en la secreción de mucinas y viéndose reducida drásticamente su actividad protectora tras el pretratamiento con NEM (Mezzaroba et al., 2006). Partiendo del paralelismo observado para los dos hidrolizados estudiados entre la actividad productora de mucinas *in vitro* y la actividad antiulcerogénica *in vivo*, la evaluación de las propiedades gástrico-protectoras de los péptidos, que han demostrado también efecto estimulante en la síntesis de mucinas, podría representar la descripción de los primeros péptidos alimentarios con capacidad inhibitoria frente a la formación de úlceras gástricas.

3.2. Actividad inhibidora de la enzima dipeptidil peptidasa-IV

La diabetes mellitus tipo 2 se considera, junto a la obesidad, una de las grandes epidemias del siglo XXI. Se caracteriza por un aumento de los niveles plasmáticos de glucosa y una progresiva pérdida de actividad de las células pancreáticas β, responsables de la secreción de insulina (Bharatam et al., 2007). Afortunadamente, existen tratamientos orales que muestran un efecto prometedor frente a esta enfermedad, como es la administración de inhibidores de la enzima DPP-IV (Namba et al., 2013). Esta enzima es la mayor responsable de la rápida degradación *in vivo* de las incretinas, hormonas generadas en el intestino tras la ingesta de alimentos y encargadas de la secreción de la insulina por parte de las células pancreáticas β (Wu et al., 2009). Se ha comprobado que la administración de inhibidores de la DPP-IV repercute en una mayor vida media de GIP y GLP-1 circulantes, las principales incretinas implicadas en la síntesis de insulina, consiguiendo así un descenso en la glucosa plasmática (Kshirsagar et al., 2011).

En este trabajo (Publicación VI) se propuso evaluar el efecto de la β-lactoglobulina como fuente de péptidos con capacidad inhibidora de la DPP-IV. Su justificación está basada en los resultados obtenidos en estudios clínicos y preclínicos sobre las propiedades de las proteínas de suero, que logran aumentar la tolerancia a la glucosa, presuntamente mediante la inhibición de la DPP-IV (Gunnarsson et al., 2006; Sousa et al., 2012). La α-lactoalbúmina, así como un hidrolizado de esta proteína con tripsina, no mostraron efecto inhibitorio de esta enzima, al contrario que un hidrolizado de β-lactoglobulina que sí lo mostró, exhibiendo además un descenso significativo del nivel de glucosa en un test de tolerancia en ratones (Uchida et al., 2011). En otro ensayo *in vivo*, un suplemento basado en un hidrolizado de proteínas de suero mostró un nivel superior de insulina respecto al obtenido por un aislado de

proteína de suero, por lo que el efecto de los péptidos derivados de dichas proteínas parece demostrado (Toedebusch et al., 2012).

En nuestro estudio, el hidrolizado de WPC enriquecido en β-lactoglobulina, que ha demostrado actividad estimulante sobre la producción de mucinas en células caliciformes (Publicación II) y antiulcerogénica en un modelo de etanol absoluto en ratas (Publicación V), también mostró actividad inhibitoria de la DPP-IV (Publicación VI). No obstante, no se observó ninguna actividad inhibitoria para el WPC sin hidrolizar, por lo que péptidos contenidos en el hidrolizado fueron los responsables del efecto inhibitorio. El valor de IC_{50} obtenido para el hidrolizado (1,51 mg mL^{-1}), aunque algo superior al descrito para un hidrolizado con pepsina de un aislado de proteínas de suero (0,075 mg mL^{-1}) (Lacroix & Li-Chan, 2012b), representa una actividad comparable al obtenido para otros hidrolizados descritos como activos. Nongonierma et al. (2013a; 2013b) midieron valores de IC_{50} comprendidos entre 0,8 y 1,4 mg mL^{-1} para distintos hidrolizados, derivados de fuentes proteicas como caseína, proteína de suero o lactoferrina. La patente WO 2009/128713 (Aart et al., 2009) recoge también la actividad encontrada para hidrolizados de caseína y lisozima con distintas enzimas proteolíticas, observándose una clara influencia del tipo de enzima sobre la actividad resultante. En dicha patente, los hidrolizados de caseína mostraban valores de IC_{50} en el rango de 2,7-5,0 mg mL^{-1} y los correspondientes de lisozima entre 0,4 y 1,5 mg mL^{-1}, dependiendo de la enzima empleada. El efecto de la enzima sobre la actividad inhibitoria final puede ser muy significativo, como es el caso de un hidrolizado de salvado de arroz variando su IC_{50} entre 2,3 y 26,4 mg mL^{-1} al emplear las enzimas comerciales Umamizyme G o Bioprase GP, respectivamente (Hatanaka et al., 2012).

Los péptidos descritos en la bibliografía con capacidad inhibidora de la DPP-IV presentan una longitud entre 2-8 aminoácidos y una naturaleza predominantemente hidrofóbica. La segunda posición amino terminal juega un papel crucial, donde la prolina parece desempeñar la mayor actividad, seguido de alanina y glicina, y en

menor medida otros aminoácidos pequeños y no cargados (Kuhn-Wache et al., 2011). En el caso de dipéptidos X-P la acción inhibitoria se ve profundamente influida por el péptido en posición amino terminal, observándose que los más activos son isoleucina, valina y metionina (Pieter et al., 2006; Hatanaka et al., 2012).

El hidrolizado objeto de estudio se separó mediante HPLC en seis fracciones, mostrando dos de ellas una clara actividad inhibidora en comparación al resto. La fracción más activa presentó un IC_{50} de 86,0 μg mL^{-1}. Se caracterizaron las dos fracciones más activas, identificándose un total de 15 secuencias peptídicas, evaluándose la actividad inhibitoria en seis de ellas seleccionadas en función de su secuencia. IPAVF e IPAVFK fueron seleccionados por presentar una prolina en segunda posición amino terminal y una isoleucina en el extremo amino terminal, estructura muy favorable que explica la actividad demostrada ya por el péptido IPA (Tulipano et al., 2011). TPEVDDEALEK también presenta una prolina en segunda posición y los péptidos LIVTQTM, VLVLDTDYK y ALPMHIR fueron elegidos en base a su abundancia relativa y la existencia de aminoácidos hidrofóbicos en segunda posición amino terminal. Los péptidos IPAVF e IPAVFK mostraron una gran capacidad de inhibición frente a la DPP-IV, con un valor de IC_{50} para IPAVF ligeramente inferior al descrito para IPA (Tulipano et al., 2011) (44,7 vs 49 μM), con lo que se podría plantear la hipótesis de que la presencia de la valina y la fenilalanina pudieran estar mejorando el efecto. Sin embargo, la existencia de la lisina en el extremo carboxi terminal disminuyó la inhibición observada hasta un valor de IC_{50} de 143,0 μM, hecho que podría quedar fundamentado en la disminución del carácter hidrofóbico del péptido en comparación con su secuencia derivada IPAVF. No obstante, esta misma situación se da entre LPVPQ y LPVPQK aunque con inverso comportamiento, ya que la secuencia con lisina demostró aproximadamente el doble de actividad que la forma sin ella, alcanzando un 63% de la actividad inhibitoria expresada por la diprotina A (Pieter, 2006). Otro caso interesante es el de un péptido derivado de la β-caseína, LPQNIPPL,

identificado en un queso Gouda y que ha llegado a demostrar disminución *in vivo* de los niveles plasmáticos de glucosa, registrándose un valor de IC_{50} cuatro veces inferior al de su secuencia derivada LPQNIPP (46 vs 160 μM) (Uenishi et al., 2012). Las secuencias TPEVDDEALEK y VLVLDTDYK demostraron un moderado efecto inhibidor de la DPP-IV, alcanzando valores de IC_{50} de 319,5 y 424,4 μM, respectivamente, lo que representa un importante descenso de actividad frente a IPAVF e IPAVFK, y que podría estar influido especialmente en el primer caso por la considerable longitud de la secuencia. Ni LIVTQTM ni ALPMHIR mostraron efecto inhibitorio apreciable sobre la actividad de la DPP-IV. Previamente, se había descrito que la secuencia derivada ALPMH no reducía significativamente la actividad de la DPP-IV (Tulipano et al., 2011), pero la presencia de la leucina y prolina en segunda y tercera posición y la variable influencia de la longitud de la secuencia, observada en otros casos, no impedía la posibilidad de que ALPMHIR pudiera llegar a presentar alguna actividad inhibitoria. En la caracterización de la segunda mejor fracción también se identificó el péptido VAGTWY, que previamente había mostrado propiedades inhibitorias de la DPP-IV (Uchida et al., 2011). Concretamente, dicho péptido fue responsabilizado de la disminución del nivel plasmático de glucosa mostrado en ratones por parte de un hidrolizado de β-lactoglobulina desarrollado con tripsina durante 24 h. En nuestro caso, la destacada actividad mostrada por una de las fracciones, puede que se deba principalmente a la acción de las secuencias IPAVF e IPAVFK, las cuales producen las mayores inhibiciones y se presentan como unas de las más abundantes en la fracción cromatográfica más activa del hidrolizado.

Se espera que el transporte transepitelial de una gran parte de los di- y tripéptidos con actividad inhibitoria de la DPP-IV se pueda llegar a alcanzar sin alteración de los mismos (Satake et al., 2002). No obstante, en el caso de péptidos más largos, podría ser interesante ver su efecto en digestiones simuladas o en ensayos de biodisponibilidad celular, ya que podría darse la situación de que la acción

de las enzimas proteolíticas gastrointestinales conlleven la liberación de otras secuencias en las que la actividad sea menor a la obtenida *in vitro* o, por el contrario, se generen fragmentos más activos. Por ejemplo, en el caso de unos péptidos derivados de atún con masas moleculares comprendidas entre 1300 y 1700 Da, y valores de IC_{50} entre 78 y 116 μM, la digestión simulada de los mismos mantuvo o incluso incrementó el efecto inhibitorio asociado (Huang et al., 2012). Sería interesante en futuros trabajos el estudio de simulados gastrointestinales del hidrolizado de WPC, así como de las fracciones o péptidos con actividad inhibitoria de la DPP-IV, para posteriormente pasar al estudio *in vivo* de los mismos.

4. CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

1. Novel food peptides with stimulatory activity on the secretion of mucins in HT29-MTX human intestinal goblet-like cells have been identified. These corresponded to the following peptides with proved or probable opioid activity: amidated form of α-lactorphin (YGLF-NH_2), the amidated and non-amidated forms of β-lactorphin (YLLF / YLLF-NH_2), α_{s1}-casein fragments 90-94 (RYLGY), 143-149 (AYFYPEL), and 144-149 (YFYPEL), human β-casomorphin 5 (YPFVE), and gluten exorphin A5 (GYYPT).

2. Among these peptides with effect on mucin secretion, the amidated forms of α-lactorphin (YGLF-NH_2) and β-lactorphin (YLLF-NH_2) as well as α_{s1}-casein fragments 143-149 (AYFYPEL) and 144-149 (YFYPEL) also induced mucin 5AC gene expression in HT29-MTX cells.

3. α_{s1}-Casein fragments 144-149 (YFYPEL) and 144-148 (YFYPE) demonstrated, for the first time, opioid activity in experiments with guinea-pig ileum preparations.

4. A peptic casein hydrolysate and a tryptic hydrolysate from a whey protein concentrate (WPC) enriched in β-lactoglobulin, containing the previously mentioned peptides, induced mucin secretion and mucin 5AC expression in HT29-MTX cells. The mucin stimulatory activity of these hydrolysates was higher than that predicted in view of their concentration of active peptides, suggesting the participation of other compounds or a synergistic effect.

5. The WPC and casein hydrolysates with mucin stimulatory activity showed antiulcerative activity in an ethanol-induced ulcer model in rats. In the case of the WPC hydrolysate, the protective effect was partly reverted by the blockage of active sulfhydryl groups.

6. The WPC hydrolysate inhibited the activity of the dipeptidyl-peptidase IV enzyme (DPP-IV). Among the identified peptides, the β-lactoglobulin fragments 78-82 (IPAVF) and 78-83 (IPAVFK) exerted a potent DPP-IV inhibitory activity which could contribute to the regulation of postprandial insulin levels.

CONCLUSIONES

1. Se han identificado nuevos péptidos de origen alimentario con actividad estimulante sobre la secreción de mucinas en células caliciformes intestinales humanas HT29-MTX. Estos péptidos se corresponden con las siguientes secuencias, con probada o probable actividad opioide: α-lactorfina en su forma amidada (YGLF-NH_2), las formas amidada y no amidada de la β-lactorfina (YLLF / YLLF-NH_2), los fragmentos 90-94 (RYLGY), 143-149 (AYFYPEL) y 144-149 (YFYPEL) de la α_{s1}-caseína, la β-casomorfina 5 humana (YPFVE) y la exorfina A5 de gluten (GYYPT).

2. De los péptidos con efecto estimulante sobre la secreción de mucinas, las formas amidadas de la α-lactorfina (YGLF-NH_2) y la β-lactorfina (YLLF-NH_2), así como los fragmentos 143-149 (AYFYPEL) y 144-149 (YFYPEL) de la α_{s1}-caseína, produjeron, además, un aumento de la expresión génica de la mucina 5AC en las células HT29-MTX.

3. Los fragmentos 144-149 (YFYPEL) y 144-148 (YFYPE) de la α_{s1}-caseína demostraron, por primera vez, actividad opioide en experimentos con íleon de cobayo.

4. Un hidrolizado de caseína con pepsina y un hidrolizado de un concentrado de proteínas de suero (WPC) enriquecido en β-lactoglobulina con tripsina, que contenían los péptidos mencionados previamente, indujeron la secreción de mucinas y la expresión génica de la mucina 5AC en las células HT29-MTX. La actividad estimulante de dichos hidrolizados fue mayor que la prevista en base a la concentración de los péptidos activos, lo que sugiere la participación de otros componentes o un efecto sinérgico.

5. Los hidrolizados de caseína y WPC con actividad estimulante sobre la producción de mucinas mostraron también actividad antiulcerogénica en un modelo de úlceras inducidas por etanol en ratas. En el caso del hidrolizado de WPC, el efecto protector revirtió parcialmente al bloquear los grupos sulfhidrilos activos, lo que sugiere que estos están implicados en el mecanismo protector del hidrolizado de WPC.

6. El hidrolizado de WPC inhibió la actividad de la enzima dipeptidil-peptidasa IV (DPP-IV). Entre los péptidos identificados, los fragmentos 78-82 (IPAVF) y 78-83 (IPAVFK) de la β-lactoglobulina ejercieron una potente actividad inhibitoria de la DPP-IV, por lo que podrían contribuir a la regulación de los niveles postprandiales de insulina.

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6. ANNEXES / ANEXOS

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Antihypertensive effect of a bovine lactoferrin pepsin hydrolysate: Identification of novel active peptides

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ABSTRACT

The potential of bovine lactoferrin (LF) as a source of antihypertensive peptides has been examined. For this purpose, LF pepsin hydrolysate with molecular mass lower than 3 kDa (LFH < 3 kDa) was prepared and orally administered to spontaneously hypertensive rats (SHR), resulting in reduced systolic blood pressure in a significant and maintained manner up to 24 h after administration. LFH < 3 kDa was further fractionated by semi-preparative high performance liquid chromatography (HPLC) and 38 peptides, contained in the active fractions, were identified by using an ion trap mass spectrometer. Based on the peptide abundance, a total of 11 peptides were chemically synthesized and their ACE inhibitory activity tested. Only three of them, corresponding to peptides of sequences LIWKL, RPYL and LNNSRAP exerted *in vitro* inhibitory effects on angiotensin I converting enzyme (ACE) activity and had a 50% inhibitory concentration (IC_{50}) of 0.47, 56.5 and 105.3 μM, respectively. The three peptides also showed antihypertensive effects in SHR and remarkably the effect of LIWKL remained significant for up to 24 h post-administration, similarly LFH < 3 kDa and the captopril control. The two most potent *in vitro* inhibitory peptides showed *ex vivo* inhibitory effect on ACE-dependent vasoconstriction as well. In conclusion, three novel LF-derived peptides and a pepsin LFH < 3 kDa lowered blood pressure and exhibit potential as orally effective antihypertensive compounds.

1. Introduction

Over the past two decades increasing scientific and industrial interest has been focused on the biological properties of milk proteins, which possess additional physiological effects due to the numerous bioactive peptides that are encrypted within intact proteins. Those peptides, once released, exhibit different activities affecting the digestive, cardiovascular, immune and nervous systems (Korhonen & Pihlanto, 2006). Among them, those with blood pressure-lowering effects are receiving increasing attention due to the prevalence and importance of hypertension in the Western population (Ricci, Artacho, & Olalla, 2010). The antihypertensive effect of milk protein derived peptides seems to be mainly due to the inhibition of angiotensin I-converting enzyme (ACE), a key enzyme in the renin-angiotensin system which plays an important role in the regulation of systemic blood pressure. ACE hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive peptide (Campbell, 2003) leading to blood pressure upregulation.

In this context, technological processes for the isolation and enrichment of bioactive peptides have been developed and proven effective. Basically, processing of milk proteins with: (i) food grade proteolytic preparations of plant, fungal or microbial origin or digestive enzymes or (ii) fermentation of milk with proteolytic starters have been employed to release ACE inhibitory peptides. Both approaches have conducted due to the development of commercial products based on casein or whey proteins with antihypertensive effects in humans (De Leo, Panarese, Gallerani, & Ceci, 2009). In fact, the most popular functional foods contain the casein-derived ACE-inhibitory tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), obtained by means of either milk fermentation

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(Nakamura, Yamamoto, Sakai, & Takano, 1995) or enzymatic hydrolysis using microbial proteases (Mizuno, Nishimura, Matsuura, Gotou, & Yamamoto, 2004).

In contrast to other milk proteins, there is scarce information about the potential of bovine lactoferrin (LF), a well-characterized component of milk whey, as a source of antihypertensive peptides. Recently, we have shown the vasoactive effects through ACE inhibition of a set of peptides derived from lactoferricin B (LfcinB), the well-known antimicrobial LF-derived peptide (Gifford, Hunter, & Vogel, 2005). We also described their efficacy as orally effective antihypertensive peptides (Centeno et al., 2006; Ruiz-Giménez et al., 2010). Moreover we have reported the inhibitory effects of a LF pepsin hydrolysate on ACE activity and ACE-dependent vasoconstriction using *in vitro* and *ex vivo* functional assays, respectively (Ruiz-Giménez et al., 2007). However, the antihypertensive effect of LF-derived peptides is not fully understood and according to previous studies, some other antihypertensive peptides are expected to be still identified and isolated from LF hydrolysate (Lee, Cheng, Enomoto, & Nakamura, 2006; Vermeirssen, van der Bent, van Camp, van Amerongen, & Verstraete, 2004).

The objective of the present study was to investigate the potential of LF as a source of antihypertensive peptides other than LfcinB-derived peptides. For this purpose, a LF pepsin hydrolysate (LFH) was ultrafiltered and fractionated. The ACE-inhibitory effect of the whole LFH (<3 kDa) permeate, as well as of each collected fraction, was measured. The main peptides from the active fractions were identified by using HPLC coupled to tandem mass spectrometry and chemically synthesized. Selected peptides were evaluated for their inhibitory effects on ACE activity and ACE-dependent vasoconstriction. The antihypertensive effects of individual ACE-inhibiting peptides, LFH and LFH (<3 kDa) permeate were assessed in spontaneously hypertensive rats (SHRs). Finally, the stability of ACE-inhibiting antihypertensive peptides against gastrointestinal digestion and intestinal peptidases was assessed. Both the hydrolysate and individual peptides could be applied as nutraceuticals in the context of health-promoting functional foods for the treatment of hypertension.

2. Materials and methods

2.1. Lactoferrin hydrolysis and fractionation of the hydrolysate

Bovine LF (USB Corp., Cleveland, Ohio, OH, USA) was dissolved in distilled water at 5% (w/vol; pH 2.5) and hydrolysed using porcine pepsin (3 % w/w, 2540 units/mg solid; Sigma Chemical Co., St. Louis, MO, USA). The hydrolysis reaction was performed at 37 °C for 4 h, as described previously (Ruiz-Giménez et al., 2007).

LFH was subjected to ultrafiltration through a VivaFlow 50 with a 3 kDa cut-off polyethersulfone membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France) and the permeate fractionated by reverse phase high performance liquid chromatography (RP-HPLC) on a Waters system (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector, a 717 plus Autosampler in combination with a Fraction Collector III. The 3 kDa permeate was applied to a Prep Nova-Pak® HR C18, 60 Å, 6 μm, 7.8 × 300 mm column (Waters). The column was developed at a flow rate of 4 ml/min. Elution was performed with a linear gradient of solvent B (acetonitrile with 0.05 % TFA) in solvent A (water with 0.05 % TFA) from 0 to 30 % B in 70 min. Samples of the whole permeate and the fractions (20 ml) were freeze-dried and kept at −20 °C until reconstitution with distilled water for determination of the protein content and *in vitro* ACE inhibitory effect, as explained later on.

2.2. In vitro assay of ACE inhibitory effect

In vitro ACE inhibitory activity of LFH < 3 kDa, fractions and synthetic peptides was measured using the fluorescent method described by Sentandreu and Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate *o*-aminobenzoyl-glycyl-*p*-nitrophenylalanylproline (Bachem Feinchemikalien, Bubendorf, Switzerland) by the action of ACE (porcine kidney, Sigma). Protein content of peptide fractions was estimated by the bicinchoninic acid method (BCA; Sigma) using bovine serum albumin as the standard.

The IC_{50} value was defined as the peptide concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by non-linear regression of the experimental data to a four-parameter logistic curve using the software package SigmaPlot v 8.02 (SPSS Inc., Chicago, IL).

2.3. Peptide sequencing by RP-HPLC-MS/MS

RP-HPLC-MS/MS analysis of the ACE-inhibiting fractions was performed on an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany) with a mediterranea^{TM}sea$_{18}$ column (150 × 2.1 mm, 5 μm of particle size; Teknokroma, Barcelona, Spain). The flow rate was 0.2 ml/min and the injection volume 50 μl. Peptides were eluted with a linear gradient of solvent B (acetonitrile with 0.027% TFA) in A (acetonitrile with 0.037% TFA) going from 0% to 20% B over 15 min followed by a 40 min gradient from 20% to 45%. The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GMBH, Bremen, Germany) equipped with an electrospray ionization source, as previously described (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Data Analysis (version 4.0; Bruker Daltoniks) was used to process and transform spectra to their representive mass values. BioTools (version 3.1; Bruker Daltoniks) was used to process the MSn spectra, to perform peptide sequencing and to calculate theoretical masses.

The main peptides identified in the ACE-inhibiting fractions were ordered at >90% purity from GenScript Corporation (Piscataway, NJ), wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. The synthetic peptide concentration was based on the dry weight of the peptides. These peptides were subjected to a four-step screening study to assess (1) *in vitro* inhibitory effect on ACE activity, (2) *ex vivo* inhibitory effect on ACE-dependent vasoconstriction, (3) *in vivo* antihypertensive effect and (4) *in vitro* resistance to simulated gastrointestinal digestion and intestinal stability.

Fragments derived from LIWKL were identified by RP-HPLC-MS/MS (ProteoRed, Proteomics Facility, Centro de Investigación Príncipe Felipe, Valencia, Spain).

2.4. Evaluation of potential antihypertensive peptides

2.4.1. Animal welfare

Rabbits and rats were housed in temperature-controlled rooms (23 °C) with 12 h light/dark cycles, where they could consume tap water and standard diets *ad libitum*. The experimental procedures performed conformed to the Spanish legislation on 'Protection of Animals used for Experimental and other Scientific Purposes' and to the Directives of the European Community on this subject.

2.4.2. Ex vivo functional assay of ACE inhibitory effect

Six male New Zealand White rabbits (Technology Transferring Center, Polytechnic University of Valencia, Valencia, Spain), weighing 2.5–3 kg, were killed by injection of 25 mg/kg sodium thiopental (Tiobarbital Braun, B. Braun Medical, Jaén, Spain) and 1.5 ml of 10 mM KCl solution through the ear vein. A midline throat incision

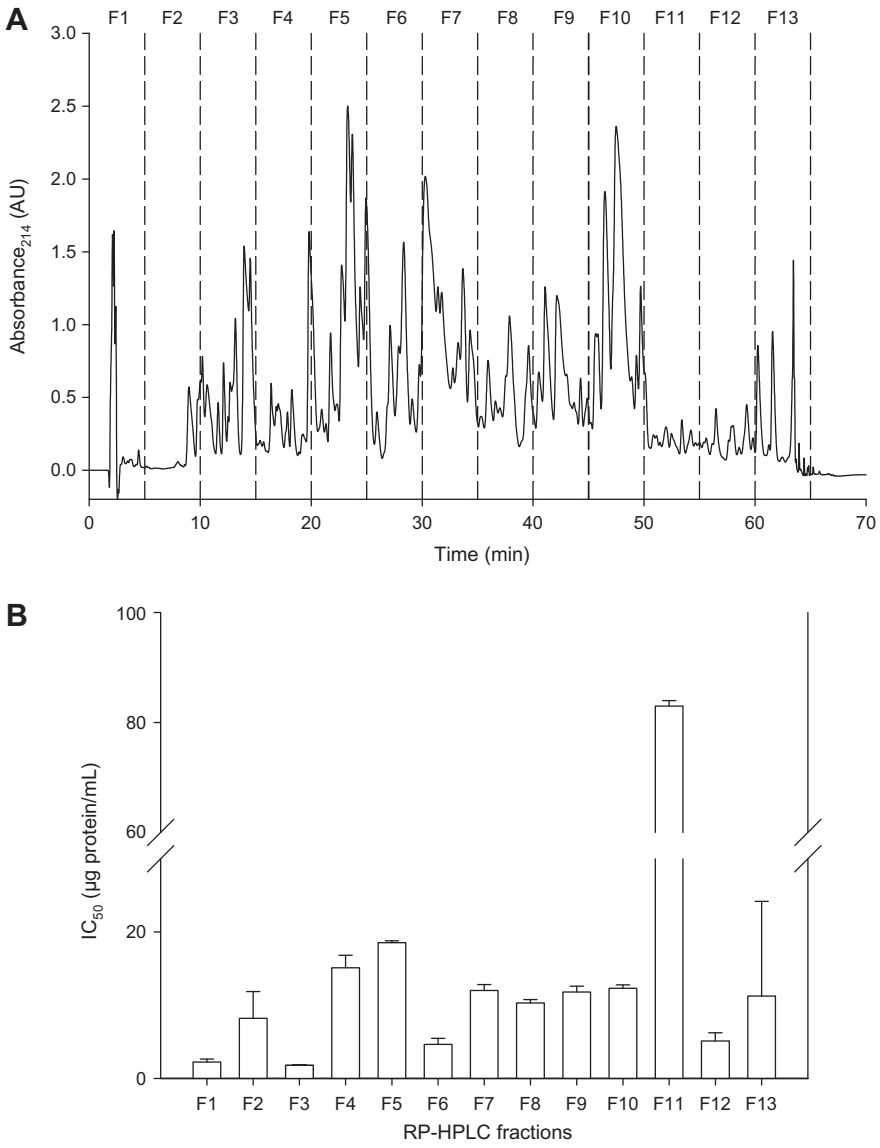

Fig. 1. Fractionation by RP-HPLC of LFH < 3 kDa (panel A) and inhibitory potency on ACE, expressed as the IC_{50} value, of the collected fractions (F1–F13) (panel B). Data are expressed as mean ± SEM for a minimum of three experiments.

provided access to both common carotid arteries, which were dissected free and cut into four 4 mm-long segments. For computer-assisted isometric tension recording, the arterial segments were mounted in an organ bath containing Ringer-Locke solution (120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM $NaHCO_3$, and 5.6 mM glucose), as described previously (Centeno et al., 2006).

The contractile capacity of every arterial segment was checked by exposure to 50 mM KCl Ringer-Locke solution (NaCl replaced with an equimolar amount of KCl). Carotid arteries contracting less than 2 g were discarded. Afterwards, every arterial segment was challenged with a single concentration (1 μM) of angiotensin I. After washing out, each arterial segment was subjected to one of the following protocols: (i) control, a second challenge to angiotensin I to check for response reproducibility or (ii) treated, preincubation (20 min) with one of the LF-derived peptides (20 μM) and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction (see Fig. 2).

2.4.3. In vivo assay of antihypertensive effect

Twenty male SHRs weighing 300–350 g were used (Charles River Laboratories, Barcelona, Spain). Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the non-invasive tail-cuff method using computer-assisted NIPREM 645 equipment (Cibertec, Madrid, Spain) as previously described (Ruiz-Giménez et al., 2010). LFH (200 mg/kg), LFH < 3 KDa (200 mg/kg) and individual LF-derived peptides (10 mg/kg) were orally administered by gastric intubation in 1 ml of physiological saline. The SBP was measured before peptide intake (zero time), in addition to, 1, 2, 3, 4 and 24 h after intake. Physiological saline alone (1 ml) and captopril (50 mg/kg) served as negative and positive controls, respectively. Each value of SBP was obtained by averaging three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the difference between the averaged values of measurements obtained before and after peptide administration.

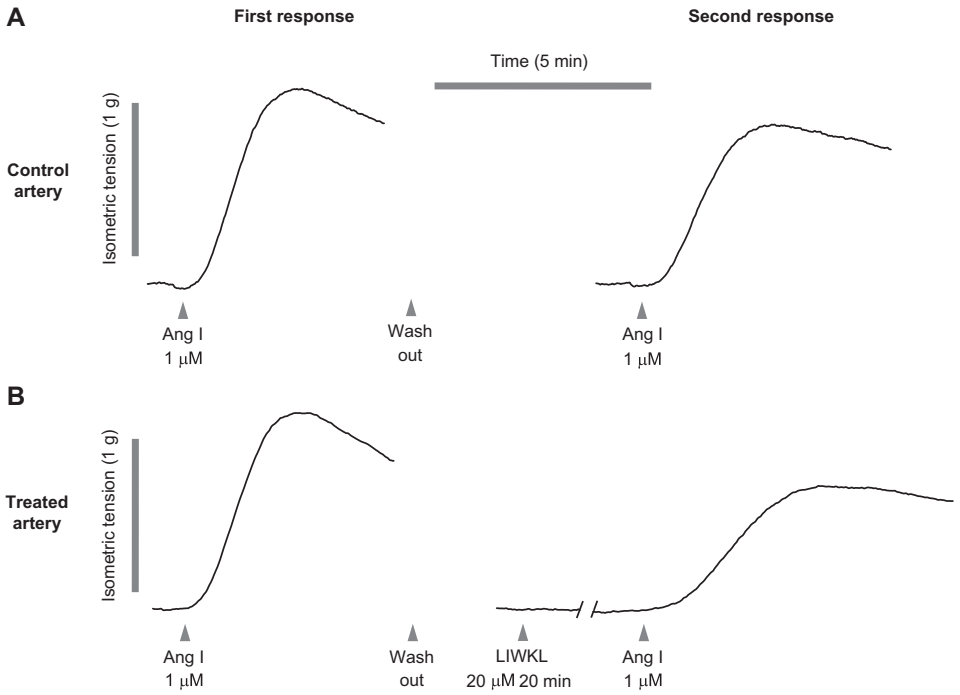

Fig. 2. Contraction of carotid artery segments. (A) Reproducible vasoconstriction to angiotensin I. (B) Effect of LIWKL on angiotensin I-induced contraction.

2.5. In vitro simulated gastrointestinal digestion and intestinal stability experiments

Peptides (1 mM) were subjected to a two-stage simulated gastrointestinal digestion process followed by a brush border peptidase phase as previously described (Ruiz-Giménez et al., 2010). Digests were stored at −20 °C until further analysis of their ACE inhibitory activity and identification of ACE inhibiting fragments.

3. Results

3.1. ACE inhibitory activity of lactoferrin hydrolysate and identification of major peptides

LFH was subjected to ultrafiltration through a 3 kDa cut-off membrane and the ACE inhibitory activity of the resulting permeate (LFH < 3 kDa) was measured, showing an IC_{50} value of 14.3 ± 3.3 μg/ml.

LFH < 3 kDa was subjected to semi-preparative RP-HPLC and the total chromatogram was divided into 13 fractions (Fig. 1A). Four fractions, F1, F3, F6 and F12, exhibited the highest ACE-inhibitory activities with IC_{50} values lower than 5 μg/ml. With the exception of F11, the rest of the chromatographic fractions showed potent ACE-inhibitory activity with IC_{50} values lower than 20 μg/ml (Fig. 1B). The four most active fractions were analysed by HPLC-MS/MS and the major peptide components were sequenced (38 peptides on total, Table 1).

3.2. ACE inhibitory activity of LF-derived peptides

A total of 11 peptides (labelled in Table 1) from those identified in fractions F1, F3, F6 and F12 were chemically synthesized and their ACE inhibitory activity was tested at a 20 μM concentration. These synthesized peptides included 10 out of the 11 most abundant peptides. Although the sequence GSRY was abundant in the F3 fraction, it was not evaluated because it is included within sequence YLGSRY from the F6 fraction. Both peptides share the last four residues at the C-terminal end, which is known to play a predominant role in competitive binding to the active site of ACE. Most of ACE inhibitory peptides share common structural features; they are short in length and their potency is strongly influenced by their C-terminal tripeptide sequence which usually contains hydrophobic amino acids and also proline, lysine or arginine residues (Murray & FitzGerald, 2007). Since the presence of a proline residue specifically at the antepenultimate position, appears to enhance peptide binding to ACE (Rohrbach, Willians, & Rolstad, 1981), the identified peptide RPYL (fraction F6) was also included in the study despite it not being abundant. Only three among the eleven peptides tested showed detectable inhibitory activity at 20 μM under our *in vitro* assay conditions: RPYL, LIWKL and LNNSRAP. Further concentration–response curves of these allowed the determination of their IC_{50} values as summarized in Table 2. It has to be highlighted that LIWKL exhibited an IC_{50} value of 0.47 μM.

3.3. Ex vivo inhibitory effect of LF-derived peptides on ACE-dependent vasoconstriction

The three ACE-inhibitory peptides were evaluated for ACE inhibition in vascular tissue using an *ex vivo* assay. We have previously reported that angiotensin I induces ACE-dependent vasoconstriction in the rabbit isolated carotid artery, as supported by the inhibition of angiotensin I-induced, but not angiotensin II-induced contractions by the ACE inhibitor captopril (Centeno et al., 2006). In the present study, challenge of the arterial segments with depolarizing solution (KCl 50 mM) induced contraction averaging 3751 ± 160 mg (n = 41). Angiotensin I (1 μM) induced phasic, transient contractions almost completely reproducible (80 ± 4%, n = 17) in two consecutive challenges to the same arterial segment (Fig. 2A). The inhibitory effects of RPYL, LIWKL and LNNSRAP (20 μM) on ACE-mediated angiotensin I-induced vasoconstriction were assessed as shown in the representative recording of Fig. 2B. As summarized in Table 2, RPYL and LIWKL induced significant inhibitions when compared to the control. By contrast,

Table 1
Identification of peptides contained in the F1, F3, F6 and F12 RP-HPLC fractions of the bovine lactoferrin pepsin hydrolysate.

Fraction[a]	Ion for MS/MS (*m*/*z*)[b]	Calculated mass	Observed mass[c]	Protein fragment	Identified sequence
F1	605.2	604.3	604.2	*f*(444–448)	EGLTW
	554.2	553.2	553.2	*f*(225–228)	DQYE
	532.3	531.3	531.3	*f*(52–56)	**KKADA** [d]
	533.0	532.2	532.0	*f*(565–568)	NRED
	661.2	660.4	660.2	*f*(51–56)	EKKADA
	731.3	730.4	730.3	*f*(97–103)	**VVKKGSN** [d]
F3	732.3	731.3	731.3	*f*(456–462)	SCHTAVD
	503.2	502.3	502.2	*f*(53–57)	KADAV
	802.4	801.5	801.4	*f*(96–103)	AVVKKGSN
	787.4	786.5	786.4	*f*(438–444)	VVKKANE
	734.2	733.3	733.2	*f*(332–337)	RETAEE
	596.2	595.3	595.2	*f*(612–616)	**LHQQA** [d]
	675.2	674.4	674.2	*f*(271–276)	LSKAQE
	411.0	410.2	410.0	*f*(400–403)	**YTAG** [d]
	482.1	481.2	481.1	*f*(321–324)	GSRY
	508.2	507.2	507.2	*f*(538–542)	GDVAF
F6	803.4	802.5	802.4	*f*(25–31)	RMKKLGA
	772.4	771.5	771.4	*f*(328–333)	LKNLRE
	992.4	991.6	991.4	*f*(338–345)	VKARYTRRV
	1018.4	1017.5	1017.4	*f*(255–266)	RSVDGKEDL
	1091.5	1090.6	1090.5	*f*(649–658)	AKLGGRPTY
	709.3	708.4	708.3	*f*(612–617)	**LHQQAL** [d]
	635.3	634.4	634.3	*f*(133–137)	RPYLS
	535.3	534.3	534.3	*f*(148–152)	**AVAKF** [d]
	758.3	757.4	757.3	*f*(319–324)	**YLGSRY** [d]
	430.1	429.3	429.1	*f*(669–672)	IANL
	548.2	547.3	547.2	*f*(133–136)	**RPYL** [d]
F12	771.3	770.4	770.3	*f*(232–238)	**LNNSRAP** [d]
	826.5	827.5	827.5	*f*(404–411)	KCGLVPVL
	647.4	646.4	646.4	*f*(402–408)	AGKCGLV
	672.4	671.4	671.4	*f*(266–270)	**LIWKL** [d]
	997.4	996.5	996.4	*f*(300–308)	FKDSALGFL
	702.3	701.4	701.3	*f*(125–130)	WVIPMG
	706.3	705.4	705.3	*f*(633–638)	KSETKN
	576.2	575.2	575.2	*f*(176–180)	EGENQ
	554.2	553.3	553.2	*f*(286–289)	FQLF
	738.3	737.4	737.3	*f*(315–321)	**DSALYLG** [d]
	491.3	490.2	490.3	*f*(635–638)	ETKN

[a] Fractions are termed as in Fig. 1.
[b] Charge of precursor ion: 1.
[c] Calculated monoisotopic mass.
[d] Chemically synthesized peptides are labelled in bold.

although contractions to angiotensin I in arterial segments preincubated with LNNSRAP were lower than in the control response, the reduction was not significant. Notably, RPYL and LIWKL showed similar inhibitory effects on ACE-dependent vasoconstriction (14% and 22% response reduction over the control conditions, respectively) despite their 100-fold different *in vitro* potencies (IC_{50} values of 56.5 and 0.47 μM, respectively).

3.4. In vivo antihypertensive effect of LF-derived peptides in SHRs

SBP, measured by the tail-cuff method in awake SHRs, was 202 ± 2 mm Hg ($n = 45$). Oral administration of the three LF-derived ACE-inhibiting peptides at 10 mg/kg induced significant reductions in SBP as shown in Fig. 3A, together with the lack of effect of oral saline and the antihypertensive effect of captopril (50 mg/kg) for comparison. The maximum antihypertensive effects were shown at 1 h post-administration for RPYL (−18.9 ± 2.3 mm Hg), LIWKL (−25.3 ± 3.5 mm Hg), and LNNSRAP (−15.3 ± 3.7 mm Hg). The antihypertensive effect remained significant for LIWKL up to 24 h post-administration and was comparable to that of the captopril control.

The antihypertensive effect of LFH and LFH < 3 kDa was also evaluated at a dose of 200 mg/kg. According to the time-course shown in Fig. 3B, oral administration of LFH did not produce significant changes in SBP of SHRs, whereas LFH < 3 kDa significantly reduced SBP and maintained the effect up to 24 h post-administration.

Table 2
Inhibitory potency and effects of selected LF-derived peptides on ACE activity and ACE-dependent, angiotensin I-induced arterial contractions.

Sequence	Protein fragment	IC_{50} (μM)[a]	Contraction peak (%)[b]
Control		n.d.[c]	80 ± 4 (17)
RPYL	*f*(133–136)	56.5 ± 1.9	69 ± 3 (11)*
LIWKL	*f*(266–270)	0.47 ± 0.01	62 ± 3 (8)**
LNNSRAP	*f*(232–238)	105.3 ± 6.4	72 ± 4 (5)

[a] IC_{50} is the mean ± SEM of at least three independent experiments.
[b] Second contraction response to angiotensin I expressed as percentage of the first contraction in the same arterial segment. Final concentration in the assay 20 μM. Data are mean ± SEM from (n) arterial segments. *Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.01$. One-way ANOVA followed by Dunnett multiple comparison tests.
[c] Not determined.

3.5. Simulated gastrointestinal digestion and intestinal stability of LF-derived peptides

The three selected peptides were subjected to a hydrolysis process which simulates physiological digestion due to gastric and pancreatic enzymes and brush border peptidases. The analysis of

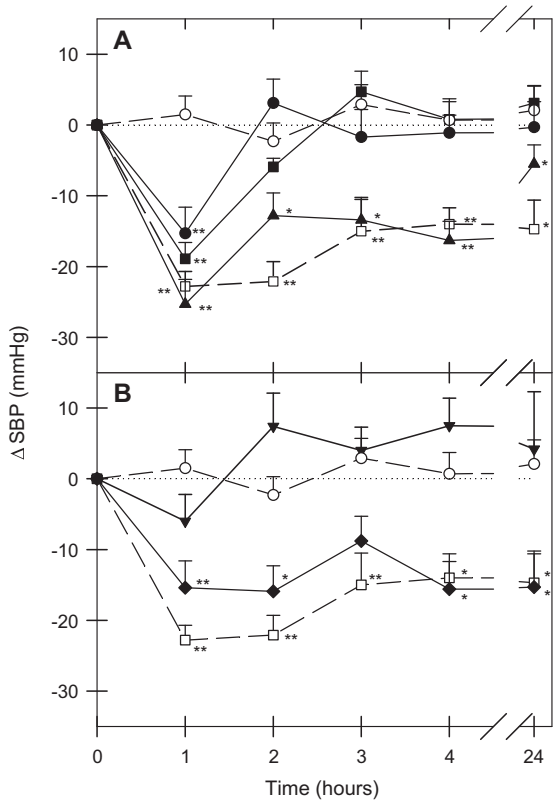

Fig. 3. Time course of systolic blood pressure (SBP) changes after oral administration of physiological saline alone (○), captopril (□, 50 mg/kg), LFH (200 mg/kg), LFH < 3 kDa (200 mg/kg) and LF-derived peptides (10 mg/kg) to SHR. Panel A: (■) RPYL; (▲) LIWKL; (●), LNNSRAP; Panel B: (▼), LFH; (♦), LFH < 3 KDa. Pressure changes (△SBP) are expressed in absolute values (mm Hg) and data are expressed as mean ± SEM for from 5–11 determinations, $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus the control group (one-way ANOVA followed by Dunnett multiple comparison tests).

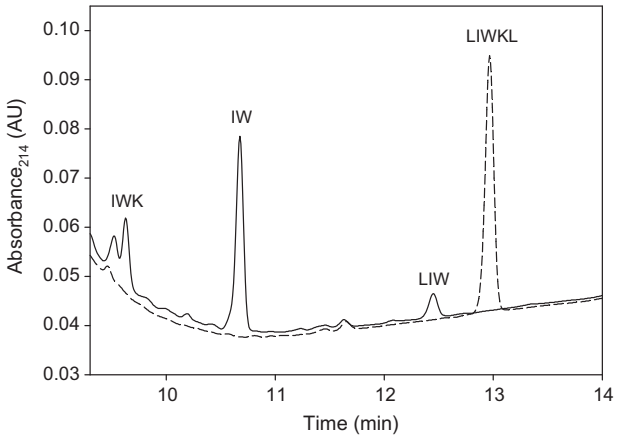

Fig. 4. RP-HPLC chromatograms of LIWKL before (dashed line) and after (solid line) being submitted to a simulated gastrointestinal digestion. Fragments IWK, IW and LIW were identified by RP-HPLC-MS/MS.

the digests by RP-HPLC (data not shown) showed that RPYL was partly hydrolyzed (approximately 65% of the initial concentration of the input peptide), whereas LIWKL and LNNSRAP were almost completely hydrolyzed (hydrolysis >90%). The resulting digests showed IC_{50} values higher than those obtained before digestion. RPYL and LNNSRAP digests showed IC_{50} values >500 μM, whereas the LIWKL digest showed a relatively potent ACE inhibitory activity (IC_{50} = 7.10 ± 1.02 μM).

The potency of the LIWKL digest prompted us to identify the peptide fragments generated by the gastrointestinal digestion and to assess their ACE inhibitory potency. Fig. 4 shows the peptide profile of the LIWKL digest and the three identified fragments: IWK, IW and LIW. The potency of two of them, IW (IC_{50} = 0.41 ± 0.02 μM) and LIW (IC_{50} = 0.22 ± 0.02 μM), was similar to that determined for the parental peptide (IC_{50} = 0.47 ± 0.01 μM), whereas the fragment IWK showed and IC_{50} value of 56.1 ± 1.9 μM.

4. Discussion

In this study we have identified three novel LF-derived peptides with antihypertensive effects after oral administration to SHRs. These peptides were isolated from a low molecular weight fraction of a LF hydrolysate (LFH) obtained by enzymatic treatment with pepsin, which produced a significant blood pressure lowering effect (−15.4 ± 3.8 mm Hg) pointing out that the antihypertensive effect may be mainly attributable to peptide components with molecular masses lower than 3 kDa, as already proposed (Mullaly, Meisel, & FitzGerald, 1997). Remarkably, the antihypertensive effect of LFH < 3 kDa remained significant up to 24 h post-administration. *In vivo* blood pressure-lowering effects have also been described for bovine casein and egg-white pepsin hydrolysates of molecular mass lower than 3 kDa although the antihypertensive effects of these products were transient and reverted 24 h after the administration (Miguel, Contreras, Recio, & Aleixandre, 2009; Miguel, López-Fandiño, Ramos, & Aleixandre, 2005).

ACE-inhibitory peptides RPYL, LIWKL and LNNSRAP isolated from the hydrolysate are reported as antihypertensive peptides for the first time. Previously, other LF-derived peptides were shown as having antihypertensive properties. A rational approach had led to the demonstration of $LfcinB_{20–25}$ (RRWQWR) and its derived fragment WQ as orally active antihypertensive peptides (Ruiz-Giménez et al., 2010). The potential of the inhibitory peptide LRPVAA (*f*(74–79)), also isolated from a pepsin hydrolysate of LF, has been suggested, although its antihypertensive effect was shown only after intravenous injection in SHR (Lee et al., 2006). None of these previous peptide sequences were identified on the present study. The sequence LIWKL share four residues with EDLIWK, a fragment isolated from a tryptic digest of LF (*f*(264–269)) and able to inhibit herpes simplex virus type 1 infection in association with ADRDQYELL (*f*(222–230)), which was isolated from the same digest (Siciliano et al., 1999). Part of this latter sequence was also identified in the fraction F1 of LFH < 3 KDa (DQYE). The multifunctional properties of milk-derived ACE-inhibitory peptides has already been pointed out since they can exert both antihypertensive and antimicrobial (Ruiz-Giménez et al., 2010) or antioxidant effects (Contreras et al., 2009).

The ACE inhibition of the <3 kDa fraction of LFH (IC_{50} = 14.3 ± 3.3 μg/mL) was comparable to the previously reported value of a casein permeate (5.68 μg/ml; Contreras et al., 2009), and as expected its *in vitro* potency was 60 times higher than the unfractionated LFH previously characterized (950 μg/ml; Ruiz-Giménez et al., 2007).

The ACE inhibitory potency of the three peptides isolated from LFH varied over a 200-fold range (Table 2). The two peptides RPYL and LNNSRAP had IC_{50} values higher than those reported for LfcinB inhibitory peptides (IC_{50} values from 2.3 to 26.7 μM; Ruiz-Giménez et al., 2010) and LRPVAA (IC_{50} = 4.14 μM; Lee et al., 2006). However, the three peptides identified in this study had more similar behaviours on *ex vivo* (Table 2) and oral administration (Fig. 2) assays, pointing out to the need of thorough full characterization of

potentially antihypertensive peptides. The lack of correlation observed between the inhibitory effects on ACE dependent vasoconstriction and the inhibitory effects observed *in vitro* on ACE activity have been also reported for lactoferricin B-derived peptides (Ruiz-Giménez et al., 2010) and casein hydrolysates (Rousseau-Ralliard et al., 2010). Although this discrepancy deserves further research, methodological detail could at least in part account for it. The *in vitro* test was carried out with porcine ACE, while rabbit arteries were used in the *ex vivo* test, and variations in the inhibition profiles of ACE from different species have been reported (Vazeux, Cotton, Cuniasse, & Dive, 2001).

LIWKL is the most potent LF-derived ACE inhibitory peptide described so far (IC_{50} = 0.47 ± 0.01 μM). Nevertheless its IC_{50} value is still far from the IC_{50} (0.022 μM) of the synthetic ACE inhibitor captopril (Ondetti, Rubin, & Cushman, 1977). LIWKL also exerted the highest antihypertensive effect on SHRs in our study (−25.3 ± 3.5 mm Hg), which is a 12.1% reduction from baseline SBP. As occurred with LFH < 3 kDa and the captopril control, the antihypertensive effect of LIWKL remained significant for up to 24 h post-administration. The peptide also showed inhibitory effects on ACE-dependent, angiotensin I-induced contractions in the carotid artery (Table 2) adding functional evidence for a mechanism of antihypertensive effect based in ACE inhibition. LIWKL was completely hydrolysed in the simulated digestion. Further data strongly suggest that the *in vivo* antihypertensive effect may be partly due to derivative fragments, since the digest showed a potent inhibitory activity (IC_{50} = 7.10 ± 1.02 μM), and two of the fragments identified (IW and LIW) showed an *in vitro* inhibitory activity as potent as that of the intact peptide. The sequence IW was among the most potent dipeptide ACE inhibitors described in the pioneering studies of Cheung, Wang, Ondetti, Sabo, and Cushman (1980). To what extent LIWKL or its derived fragments are responsible for the *in vivo* effects deserves further research.

Although RPYL and LNNSRAP exerted antihypertensive efficacy, the effect was lost at 2 h post-administration. This result might be related to gastrointestinal digestion and the release of non-active peptides as shown by the IC_{50} values of the corresponding digests. In contrast to the inhibitory effect on ACE-dependent vasoconstriction shown by RPYL, LNNSRAP did not show such an effect, suggesting a mechanism for the antihypertensive effect other than inhibition of ACE-related vasoactive effects. This result is similar to our previous data with the fragment WQ derived from the gastrointestinal digestion of $LfcinB_{20\text{-}25}$ (Ruiz-Giménez et al., 2010) and also with α_{S1}- and α_{S2}-casein hydrolysates, which failed to inhibit angiotensin I-induced vasoconstriction in aortic tissue (Rousseau-Ralliard et al., 2010). Increasing evidence is being provided that mechanisms other than ACE inhibition may also be involved in the blood pressure-lowering effect exerted by many food derived peptides (López-Fandiño, Otte, & van Camp, 2006). It has been described that a casein hydrolysate containing VPP and IPP improves vascular endothelial function independent of blood pressure lowering effects (Hirota et al., 2007). Previously, it was reported that the modulation of endothelin-1 (ET-1), a peptide that has powerful vasoconstrictor and pressor properties (Kedzierski & Yanagisawa, 2001), may play a role in the antihypertensive effects of the ACE-inhibitory milk-derived peptide lactokinin (Maes et al., 2004). Recently, we have shown that some ACE-inhibitory LfcinB-derived peptides can also act as inhibitors of the endothelin converting enzyme, the key peptidase in the production of ET-1 (Fernández-Musoles et al., 2010). In the same way, different cardiovascular benefits have been recently described after the oral administration of an antihypertensive casein hydrolysate to SHR. It improved aorta and mesenteric acetylcholine relaxations, increased the endothelial nitric oxide synthase expression in the aorta and decreased left ventricular hypertrophy, accompanied by a significant decrease in interstitial fibrosis (Sánchez et al., 2011).

5. Conclusions

We have shown that hydrolysis of LF with pepsin generates antihypertensive peptides identified as sequences RPYL, LIWKL and LNNSRAP. Also, data reported here demonstrate that LFH of molecular mass lower than 3 kDa exerts antihypertensive effects suggesting its potential application as a nutraceutical in the treatment of hypertension. In this context, it is more likely that the hydrolysate would be better accepted by the industry and consumers than the use of pure peptides, which would be more conceivable for pharmaceutical applications. Whether only the three sequences identified are responsible for the observed ACE inhibiting and antihypertensive effects of LFH < 3 kDa requires further characterization studies. Mechanisms other than ACE inhibition potentially involved in the antihypertensive effect of LF-derived peptides deserve further research.

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Antihypertensive effects of lactoferrin hydrolyzates: Inhibition of angiotensin- and endothelin-converting enzymes

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ABSTRACT

The potential of bovine lactoferrin (LF) as a source of antihypertensive peptides acting on the renin–angiotensin system (RAS) and the endothelin (ET) system as dual vasopeptidase inhibitors has been examined. For this purpose enzymatic LF hydrolyzates (LFHs) were generated by trypsin and proteinase K digestions. Permeate fractions with molecular masses lower than 3 kDa (LFH <3 kDa) were orally administered to spontaneously hypertensive rats (SHRs). Although both LFHs <3 kDa showed *in vitro* angiotensin I-converting enzyme (ACE)-inhibitory activity, only proteinase K LFH <3 kDa exerted an *in vivo* antihypertensive effect. The proteinase K LFH <3 kDa and a previously characterized pepsin LFH <3 kDa with ACE-inhibitory and antihypertensive effects were tested in *ex vivo* functional assays as inhibitors of ACE-dependent vasoconstriction. Pepsin LFH <3 kDa but not proteinase K LFH <3 kDa inhibited ACE-dependent vasoconstriction. When tested as inhibitors towards endothelin-converting enzyme (ECE), both LFHs <3 kDa exerted *in vitro* inhibitory effects on ECE activity and inhibited ECE-dependent vasoconstriction. Most abundant peptides in proteinase K LFH <3 kDa were identified by using an ion trap mass spectrometer. Based on peptide abundance, two peptides (GILRPY and REPYFGY) were chemically synthesized and their ECE-inhibitory activity was tested. Both exerted *in vitro* inhibitory effects on ECE activity. In conclusion, orally effective antihypertensive LFHs <3 kDa may act as dual vasopeptidase (ACE/ECE) or as single ECE inhibitors with different antivasoconstrictor effects depending on the protease used to release bioactive peptide sequences.

1. Introduction

The renin–angiotensin system (RAS) is the most recognized humoral system for the control of blood pressure, and its dysfunctions are involved in the pathophysiology of hypertension. Briefly, prorenin is converted to active renin by a trypsin-like enzyme. Renin cleaves angiotensinogen to form angiotensin I. Angiotensin I-converting enzyme (ACE) hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive metabolite leading to blood pressure up-regulation (Carey & Siragy, 2003). Several antihypertensive drugs target this system at different points of the cascade (Williams, 2009). Another peptidic system, the endothelin (ET) system, also has an increasingly recognized role in blood pressure regulation, and has been targeted for hypertension drug treatment (Schiffrin, 2005). In the ET system, the endothelin-converting enzyme (ECE) cleaves the biologically inactive intermediate termed big ET-1 to form ET-1 which has powerful vasoconstrictor and pressor properties. Selective ECE inhibitors have been tested in preclinical rat models of hypertension (Battistini, Ayach, Molez, Blouin, & Jeng, 2002). Moreover, present strategies in the search for novel classes of antihypertensive drugs include the development of single compounds capable of simultaneously inhibiting more than one enzymatic activity involved in hypertension pathophysiology (Battistini, Daull, & Jeng, 2005).

In recent years interest in food protein-derived peptides with antihypertensive effects has grown as an alternative to drugs in the control of systemic blood pressure and prevention of associated cardiovascular disease events. Although ACE inhibition is

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the main goal of most of the antihypertensive peptides derived from food proteins, some of them are multifunctional. In this context, enzymatic hydrolyzates of food proteins have been reported to exert both ACE-inhibitory and antioxidant activities (Rao et al., 2012; Udenigwe & Aluko, 2010; Vastag, Popovic, Popovic, Krimer, & Pericin, 2011). Also flaxseed peptide fractions inhibited both ACE and renin activities (Udenigwe, Lin, Hou, & Aluko, 2009) and jellyfish protein hydrolyzates showed ACE-inhibitory, antihypertensive and antihyperlipidaemic activities (Liu, Zhang, Zhang, & Liu, 2012). Recently antithrombotic and ACE-inhibitory properties of peptides released from bovine casein by *Lactobacillus casei* have been described (Rojas-Ronquillo et al., 2012) and two antihypertensive sequences found in a casein hydrolyzate also stimulated mucin secretion in intestinal cells (Martínez-Maqueda et al., 2012). By contrast, few studies have focused on the potential effect of food-derived peptides on the ET-system. Only pepsin digests of bonito and beef proteins were described as ECE inhibitors (Okitsu, Morita, Kakitani, Okada, & Yokogoshi, 1995). Interestingly the ACE-inhibitory peptide lactokinin derived from the milk protein β-lactoglobulin can also modulate ET-1 release by endothelial cells (Maes et al., 2004).

Recently we characterized a set of peptides derived from lactoferricin B (LfcinB), the well-known antimicrobial lactoferrin (LF)-derived peptide (Gifford, Hunter, & Vogel, 2005), as dual vasopeptidase inhibitors since they showed inhibitory effects on ACE and ECE activities (Fernández-Musoles et al., 2010; Ruiz-Giménez et al., 2010). Moreover, we have reported the *in vivo* antihypertensive effects of an ACE-inhibitory LF hydrolyzate obtained by pepsin digestion (Ruiz-Giménez et al., 2012) although the effects on the ET-system were not assessed.

The main objective of the present study was to generate multifunctional LF hydrolyzates with ACE and ECE inhibitory properties which could be applied as functional ingredients or as pharmaceutical products for the treatment of hypertension. For this purpose, we report the preparation of new LF hydrolyzates using trypsin and proteinase K, their antihypertensive effects on spontaneously hypertensive rats (SHRs) and their evaluation as dual vasopeptidase (ACE and ECE) inhibitors. Also we further characterized the ACE-inhibitory and antihypertensive pepsin digested LF hydrolyzate by assessing its inhibitory effects on ECE activity and ECE-dependent vasoconstriction. Finally, the identification of the main peptides from the antihypertensive LF hydrolyzate generated by proteinase K is carried out, and their potential bioactivities are discussed.

2. Materials and methods

2.1. Materials

Bovine LF was provided by FrieslandCampina Domo (Zwolle, The Netherlands). Porcine pepsin and trypsin (type II-S), ACE from porcine kidney, captopril, bicinchoninic acid and human ET-1 were purchased from Sigma (St. Louis, MO, USA). Angiotensin I was supplied by Calbiochem Co. (La Jolla, CA). Human big ET-1 and o-aminobenzoylglycyl-p-nitrophenylalanylproline were from Bachem Feinchemikalien (Bubendorf, Switzerland). Human ECE-1 was provided by R&D systems (Minneapolis, MN, USA). Endothelin enzyme immunoassay (EIA) kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). Sodium thiopental (Tiobarbital Braun®) was supplied by B. Braun Medical (Jaén, Spain). Recombinant proteinase K was purchased from Roche (Mannheim, Germany).

2.2. Lactoferrin hydrolysis and ultrafiltration of hydrolyzates

Bovine LF (5% w/v) was dissolved in distilled water (pH 2.5), 20 mM Tris–HCl buffer (pH 8) containing 10 mM $CaCl_2$ or 20 mM Tris–HCl buffer (pH 7.5) and hydrolyzed using porcine pepsin (3% w/w), trypsin (1% w/w) or proteinase K (1% w/w), respectively. The reactions were performed at 37 °C for 4 h (hydrolysis with pepsin; Ruiz-Giménez et al., 2007) or 37 °C for 24 h (hydrolysis with trypsin or proteinase K; Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998). After digestion, each hydrolyzate (LFH) was heated at 80 °C for 15 min to inactivate the enzymes.

LFHs were subjected to ultrafiltration through a VivaFlow 50 cassette with a 3 kDa cut-off polyethersulfone membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France). The permeates (LFHs <3 kDa) were lyophilized and kept at room temperature until reconstitution with distilled water for further analyses. Protein content was estimated by the bicinchoninic acid method using bovine serum albumin as standard (Ruiz-Giménez et al., 2012).

2.3. In vitro assay of ACE-inhibitory effect

In vitro ACE-inhibitory activity of LFHs <3 kDa was measured using the fluorescent method described by Sentandreu and Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitrophenylalanylproline by the action of ACE.

The IC_{50} value was defined as the peptide concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by non-linear regression of the experimental data to a four-parameter logistic curve using the software package SigmaPlot v 10.0 (SPSS Inc., Chicago, IL, USA).

2.4. In vitro assay of ECE-inhibitory effect

In vitro ECE-inhibitory activity of LFHs <3 kDa and individual peptides identified in the proteinase K LFH <3 kDa was measured using the method described by Fernández-Musoles et al. (2010) based on the hydrolysis of the natural substrate big ET-1 by the action of the recombinant human ECE-1. The reaction product ET-1 was quantified with the endothelin EIA kit following the supplier's instructions.

Effects on ECE activity were expressed as percent of ECE activity inhibition with respect to a control without hydrolyzate or peptide.

2.5. Animal welfare

Rabbits and rats were housed in temperature-controlled rooms (23 °C) with 12 h light/dark cycles and consumed tap water and standard diets *ad libitum*. Experimental procedures were conducted in accordance with the Spanish legislation on 'Protection of Animals used for Experimental and other Scientific Purposes' and to the Directives of the European Community on this subject. The study was approved by the 'Ethics Committee for Animal Welfare' of the Hospital 'La Fe'.

2.6. Preparation of rabbit carotid arterial segments

Thirteen male New Zealand White rabbits (Technology Transferring Center, Polytechnic University of Valencia, Valencia, Spain), weighing 2.5–3 kg, were killed by injection of 25 mg/kg sodium thiopental and 1.5 ml of 10 mM KCl solution through the ear vein. A midline throat incision provided access to both common carotid arteries, which were dissected free and each cut into four 4 mm-long segments. For computer-assisted isometric tension recording, the arterial segments were mounted in an organ bath containing Ringer-Locke solution (120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM $NaHCO_3$, and 5.6 mM glucose), as described previously (Centeno et al., 2006).

The contractile capacity of every arterial segment was checked by exposure to 50 mM KCl Ringer-Locke solution (NaCl replaced

with an equimolar amount of KCl). Carotid arteries contracting less than 1 g were discarded.

2.7. Ex vivo functional assay of ACE-inhibitory effect

Every arterial segment was challenged with a single concentration (1 μM) of angiotensin I. After washing out, each arterial segment was subjected to one of the following protocols: (i) control, a second challenge to angiotensin I to check for response reproducibility or (ii) treated, preincubation (20 min) with one of the LFHs <3 kDa (100 μg/ml) and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction (see Fig. 2).

2.8. Ex vivo functional assay of ECE inhibitory effect

Every arterial segment was challenged with a single concentration (50 mM) of KCl. After washing out, each arterial segment was subjected to one of the following protocols: (i) a challenge to big ET-1 (0.1 μM) to obtain the control contractile response, or (ii) preincubation (20 min) with one of the LFHs <3 kDa (100 μg/ml) and a challenge to big ET-1 to check for their effect on big ET-1-induced contraction (see Fig. 3).

2.9. In vivo assay of antihypertensive effect

Twenty male SHRs weighing 300–350 g were used, together with five male Wistar Kyoto (WKY) rats as the normotensive control (Charles River Laboratories, Barcelona, Spain). Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the non-invasive tail-cuff method using computer-assisted NIPREM 645 equipment (Cibertec, Madrid, Spain) as previously described (Ruiz-Giménez et al., 2010). LFHs <3 kDa (200 mg/kg) were orally administered by gastric intubation in 1 ml of physiological saline. The SBP was measured before peptide intake (zero time), and 1, 2, 3, 4 and 24 h after intake. Physiological saline (1 ml) and captopril (50 mg/kg) served as negative and positive controls, respectively. Each value of SBP was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal.

2.10. Peptide sequencing by RP-HPLC-MS/MS

RP-HPLC–MS/MS analysis of the proteinase K LFH <3 kDa was performed on an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany) with a Mediterranea™Sea$_{18}$ column (150 × 2.1 mm, 5 μm of particle size; Teknokroma, Barcelona, Spain). The flow rate was 0.2 ml/min and the injection volume 50 μl. Peptides were eluted with a linear gradient of solvent B (acetonitrile with 0.027% TFA) in A (acetonitrile with 0.037% TFA) going from 0% to 20% B over 15 min followed by a 40 min gradient from 20% to 45%. The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GMBH, Bremen, Germany) equipped with an electrospray ionization source, as previously described (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Data Analysis (version 4.0; Bruker Daltoniks) was used to process and transform spectra to representing mass values. BioTools (version 3.1; Bruker Daltoniks) was used to process the MSn spectra, to perform peptide sequencing and to calculate theoretical masses.

Main peptides identified in the proteinase K LFH <3 kDa were ordered at >90% purity from GenScript Corporation (Piscataway, NJ) wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Synthetic peptide concentration was based on the dry weight of the peptides. Sequences were tested for their *in vitro* ECE-inhibitory effect.

3. Results and discussion

3.1. ACE-inhibitory and antihypertensive effects of lactoferrin hydrolyzates

LF was hydrolyzed with trypsin or proteinase K and hydrolyzates were subjected to ultrafiltration through a 3 kDa cut-off membrane. The resulting permeates (LFHs <3 kDa) inhibited ACE with IC_{50} values of 1.3 ± 0.1 μg/ml and 6.9 ± 0.2 μg/ml for proteinase K and trypsin LFHs, respectively. ACE-inhibitory effects of LFHs were comparable to the previously reported value of a pepsin LF permeate (IC_{50} = 14.3 ± 3.3 μg/ml) which produced a significant blood pressure lowering effect after oral administration at 200 mg/kg to SHRs (Ruiz-Giménez et al., 2012).

To further study trypsin and proteinase K permeates, their antihypertensive effects were evaluated at the same dose as the previously characterized pepsin LFH <3 kDa (200 mg/kg). SBP, measured by the tail-cuff method in awake SHRs, was 183 ± 2 mm Hg (n = 18). As shown in Fig. 1A, together with the lack of effect of oral saline and the antihypertensive effect of captopril (50 mg/kg) for

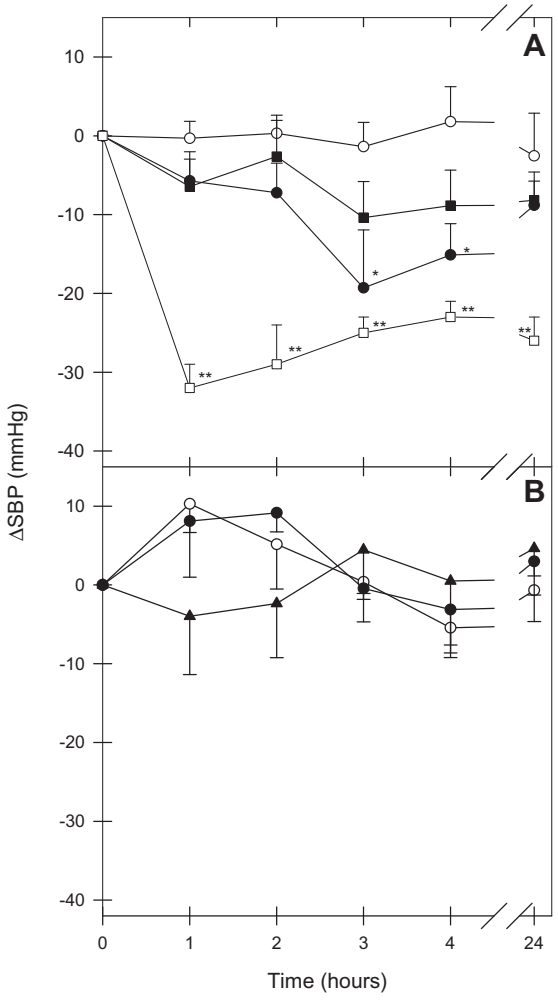

Fig. 1. Time course of systolic blood pressure changes (ΔSBP) after oral administration of enzymatic lactoferrin hydrolyzates with molecular masses lower than 3 kDa (LFHs <3 kDa). (A) Effect of physiological saline (○), captopril (□, 50 mg/kg), proteinase K LFH <3 kDa (●, 200 mg/kg) and trypsin LFH <3 kDa (■, 200 mg/kg) on SHRs. (B) Effect of physiological saline (◇), proteinase K LFH <3 kDa (●, 400 mg/kg) and pepsin LFH <3 kDa (▲, 400 mg/kg) on WKY rats. Pressure changes from baseline are expressed in absolute values (mm Hg) and data are mean ± SEM from at least 4 determinations, $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus control group (one-way ANOVA followed by Dunnett multiple comparison tests).

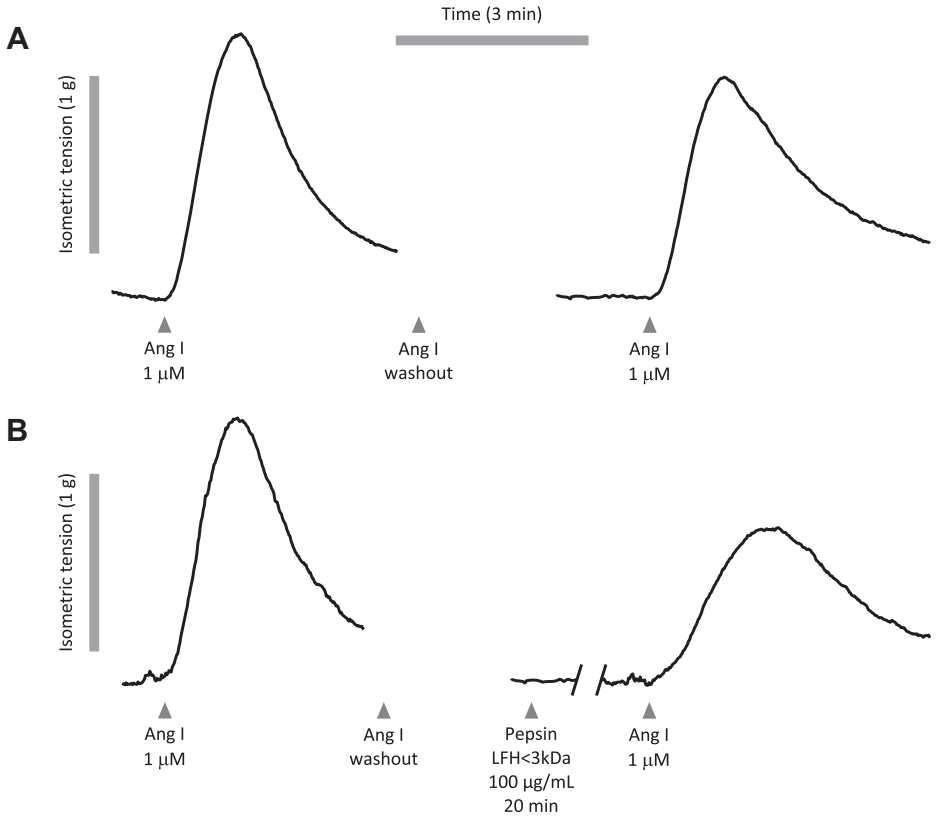

Fig. 2. Contraction of carotid artery segments. (A) Reproducible vasoconstriction to angiotensin I. (B) Effect of pepsin lactoferrin hydrolyzate with molecular mass lower than 3 kDa (LFH <3 kDa) on angiotensin I-induced contraction.

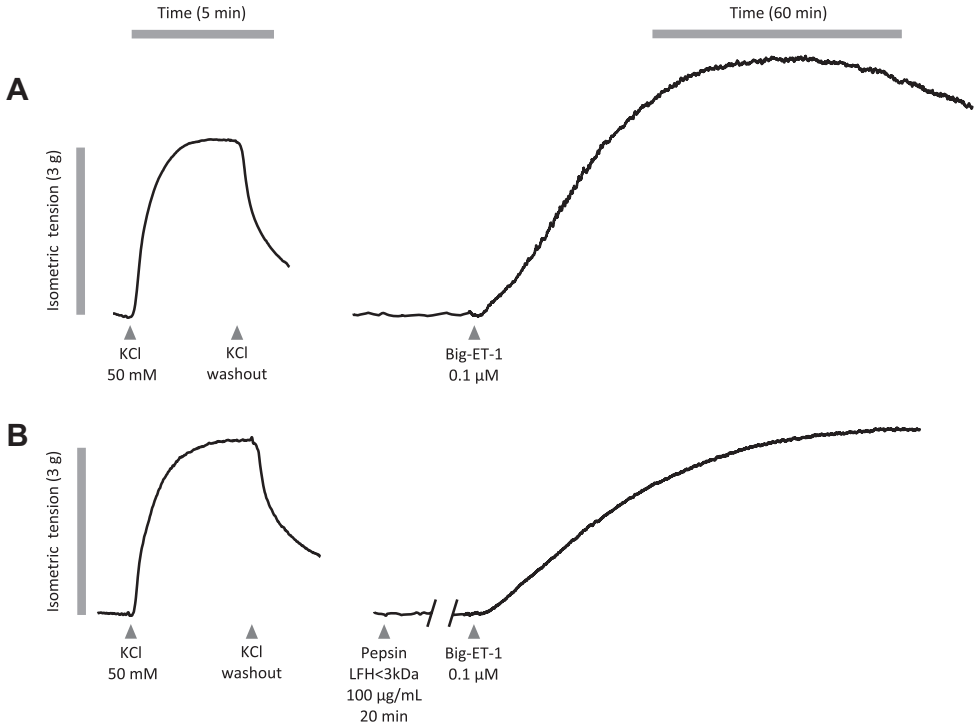

Fig. 3. Contraction of carotid artery segments. (A) Vasoconstriction to high-KCl and to big endothelin-1 (big ET-1) in control conditions. (B) Effect of pepsin lactoferrin hydrolyzate with molecular mass lower than 3 kDa (LFH <3 kDa) on big ET-1-induced contraction.

comparison, oral administration of trypsin LFH <3 kDa did not produce significant changes in SBP of SHRs. By contrast, proteinase K LFH <3 kDa significantly reduced SBP at 3 (−19 ± 7 mmHg) and 4 h (−15 ± 4 mmHg) post administration, although the effect was not maintained up to 24 h post administration as observed previously for pepsin LFH <3 kDa (Ruiz-Giménez et al., 2012). Interestingly, the antihypertensive proteinase K LFH <3 kDa and also that generated by pepsin had no effects in the SBP of the normotensive control WKY rat strain. WKY rats showed SBP of 109 ± 5 mm Hg (n = 6) and oral administration of LFHs <3 kDa at the same dose administered to SHRs and also at a higher dose (400 mg/kg) did not show significant hypotensive effects. Fig. 1B shows the lack of effects of saline and both LFHs <3 kDa after oral administration at a dose of 400 mg/kg. These results may suggest that the effects of both LFHs are specific to the hypertensive state and it could be expected a lack of arterial blood pressure effects in normotensive human subjects.

The antihypertensive effects of the LFHs <3 kDa in SHRs did not correlate with their *in vitro* ACE effects as outlined by other authors for food-derived peptides (Li, Le, Shi, & Shrestha, 2004; Vermeirssen, van Camp, & Verstraete, 2004). Abubakar et al. (1998) described digests of whey protein which showed strong ACE-inhibitory activity but no antihypertensive effect in SHRs and conversely digests with weak ACE-inhibitory activity but a strong decreasing effect of SBP. We have also described the lack of antihypertensive effect in SHRs of *in vitro* ACE-inhibitory lactoferricin B-derived peptides (Ruiz-Giménez et al., 2010). The low bioavailability of *in vitro* ACE-inhibitory peptides following oral administration may be the reason for the lack of antihypertensive effect *in vivo* and, on the contrary, peptide degradation or fragmentation during gastrointestinal digestion or vascular circulation may result in more potent ACE-inhibitory activities (Vermeirssen et al., 2004). It has been suggested that some milk derived peptides do not act directly as acute ACE inhibitors *in vivo,* but through other pathways (Fuglsang, Nilsson, & Nyborg, 2003). Another explanation is linked to the dual action of ACE which apart from converting angiotensin I in angiotensin II, inactivates bradykinin, a potent vasodilator that stimulates NO release and prostacyclin production in the endothelium (Rousseau-Ralliard et al., 2010).

3.2. Ex vivo inhibitory effects of lactoferrin hydrolyzates on ACE-dependent vasoconstriction

To add functional evidence for the ACE-inhibitory effect underlying the antihypertensive effect of proteinase K LFH <3 kDa, *ex vivo* experiments using isolated rabbit carotid arteries were carried out. Pepsin LFH <3 kDa was also included in the study to further characterize its potential mechanism of action. We have previously reported that angiotensin I induces ACE-dependent vasoconstriction in the rabbit isolated carotid artery, as supported by the inhibition of angiotensin I-induced, but not angiotensin II-induced, contractions with the ACE inhibitor captopril (Centeno et al., 2006). In the present study, challenging the arterial segments with depolarizing solution (KCl 50 mM) induced contraction averaging 3921 ± 224 mg (n = 41). Angiotensin I (1 μM) induced phasic, transient contractions almost completely reproducible (83 ± 6%, n = 13) in two consecutive challenges to the same arterial segment (Fig. 2A). The inhibitory effects of pepsin and proteinase K LFHs <3 kDa on ACE-mediated angiotensin I-induced vasoconstriction were assessed as shown in the representative recording of Fig. 2B. In a previous work, we reported significant inhibition of angiotensin I-induced vasoconstriction by a non-ultrafiltered pepsin LFH although it was only achieved at a concentration of 1350 μg/ml (Ruiz-Giménez et al., 2007). Here, as summarized in Table 1, pepsin LFH <3 kDa induced significant inhibition when compared to that of control (29% response reduction over the control conditions) at a concentration of 100 μg/ml, suggesting that the *ex vivo* ACE-inhibitory effect may be mainly attributable to peptide components with molecular masses lower than 3 kDa. By contrast, proteinase K LFH <3 kDa did not show any significant effect on ACE-dependent vasoconstriction. We have described inhibitory effects on ACE dependent vasoconstriction (response reductions up to 30% over the control conditions) for lactoferricinB-derived peptides (Ruiz-Giménez et al., 2010) and also for the peptide sequences RPYL and LIWKL (response reductions of 14% and 22%, respectively) identified in the pepsin LFH <3 kDa (Ruiz-Giménez et al., 2012).

Table 1
Effects of proteinase K and pepsin lactoferrin hydrolyzates with molecular masses lower than 3 kDa (LFHs <3 kDa) on arterial contractions induced by angiotensin I (ACE-dependent vasoconstriction) and by big ET-1 (ECE-dependent vasoconstriction).

LFH <3 kDa (100 μg/ml)	Angiotensin-I (1 μM) Contraction (%)[a]	Big ET-1 (0.1 μM) Contraction (%)[b]
Control	83 ± 6 (13)	146 ± 13 (19)
Proteinase K	79 ± 5 (13)	115 ± 4* (18)
Pepsin	59 ± 4* (15)	101 ± 7** (18)

Data are mean ± SEM from n arterial segments. *Significantly different from control, P < 0.05. **Significantly different from control, P < 0.01. One-way ANOVA followed by Dunnet's multiple comparison tests.
[a] Second contraction response to angiotensin I expressed as the percentage of the first contraction in the same arterial segment.
[b] Contraction response to big ET-1 expressed as percentage of previous KCl-induced contraction.

Similar results to those obtained here with proteinase K LFH <3 kDa were described for trypsin hydrolyzates from casein, which induced inhibition of ACE but failed to modify angiotensin I-induced aortic ring vasoconstriction (Rousseau-Ralliard et al., 2010). Although proteinase K LFH <3 kDa inhibited ACE *in vitro* and showed a moderate *in vivo* effect, *ex vivo* results suggest a mechanism for the antihypertensive effect other than ACE-dependent vasoconstriction. By contrast, the antihypertensive effect of pepsin LFH <3 kDa may be due to *in vivo* ACE inhibition and subsequent reduction of angiotensin I-induced vascular tone.

3.3. Inhibitory effects of lactoferrin hydrolyzates on ECE activity and ECE-dependent vasoconstriction

In order to investigate the potential effects of proteinase K and pepsin LFHs on the endothelin system, *in vitro* assays of ECE-inhibitory activity and functional *ex vivo* assays of inhibitory effect on ECE-dependent vasoconstriction were carried out. With respect to *in vitro* assays, both hydrolyzates showed significant ECE-inhibitory effects in a concentration-dependent manner, as summarized in Table 2. At the maximum concentration tested (500 μg/ml), proteinase K LFH <3 kDa practically abolished (98% inhibition) big ET-1 conversion, while pepsin LFH <3 kDa induced inhibition of ECE activity by approximately 60%. Only bonito and beef protein digests have been reported to exert 40–45% ECE inhibition (Okitsu et al., 1995).

To add functional evidence for the ECE-inhibitory effect underlying antihypertensive effects of proteinase K and pepsin LFH <3 kDa, both hydrolyzates were evaluated for ECE inhibition in vascular tissue using an *ex vivo* assay. We have previously reported that big ET-1 induces ECE-dependent vasoconstriction in the rabbit isolated carotid artery, as supported by the inhibition of big ET-1-induced, but not ET-1-induced contractions with the ECE inhibitor phosphoramidon (Fernández-Musoles et al., 2010). In the present study, challenge of arterial segments with a depolarizing solution (50 mM KCl) induced contraction averaging 3212 ± 179 mg (n = 55). Big ET-1 (0.1 μM) induced slow contraction with maximum effect reached at 80 ± 5 min and amounting to 146 ± 13% relative to previous responses to KCl (n = 19) (Fig. 3A). The inhibitory

Table 2
Effects of proteinase K and pepsin lactoferrin hydrolyzates with molecular masses lower than 3 kDa (LFHs <3 kDa) and selected proteinase K LF-derived peptides on endothelin-converting enzyme (ECE) activity.

	Concentration[a]	ECE inhibition[b]
Proteinase K LFH <3 kDa	500	98 ± 1**
	100	92 ± 0**
	10	71 ± 2**
Pepsin LFH <3 kDa	500	61 ± 1**
	100	31 ± 1**
	10	7 ± 4
GILRPY	300	52 ± 4**
	100	36 ± 7**
	30	13 ± 1*
REPYFGY	300	40 ± 4**
	100	29 ± 2**
	30	23 ± 3**

[a] Concentration of LFHs < 3 kDa (μg/ml) and peptides (μM) in the assay.
[b] Inhibition of ECE activity (%) expressed as mean ± SEM of at least three replicates.
* Significant inhibition respect to control, $P < 0.05$.
** Significant inhibition respect to control, $P < 0.01$ (Student's *t*-test on un-shown absolute values of ECE activity).

effects of LFHs on ECE-mediated, big ET-1-induced vasoconstriction were assessed as shown in the representative recording of Fig. 3B. As summarized in Table 1, both hydrolyzates induced inhibition when compared to control vasoconstriction, with significant reductions in the magnitude of the contraction. In a previous report, we also found inhibition of ECE-mediated vasoconstriction by several LfcinB-derived peptides when assayed at 30 μM. Moreover, we found a good positive correlation between the inhibitory effects of LfcinB-derived peptides on ECE activity observed *in vitro* and inhibitory effects on ECE-dependent vasoconstriction (Fernández-Musoles et al., 2010). Here, in contrast to *in vitro* assays of inhibitory effect, pepsin LFH <3 kDa induced higher inhibition of vasoconstriction than that produced by the proteinase K LFH <3 kDa. This apparent discrepancy could be related to methodological aspects since the *in vitro* assay was carried out with recombinant human ECE-1, while rabbit arteries were used in the *ex vivo* test. Also enzymatic pathways involved in ET-1 production by isolated vessels are more complex than those in the simple *in vitro* reaction. In fact several enzymes apart from ECE have been described to produce ET-1 from big ET-1 in rabbit aorta (Tirapelli et al., 2006). Further studies are needed to elucidate this discrepancy.

Results from ACE and ECE inhibition in vascular tissue show the inhibitory ability of pepsin LFH <3 kDa on both enzymes, suggesting dual ACE and ECE inhibition as mechanisms involved in the antihypertensive effect observed in SHRs. In contrast, ECE inhibition seems to be the mechanism involved in the moderate antihypertensive effect of proteinase K LFH <3 kDa. Recently, the antihypertensive effects in SHRs of flaxseed protein-derived arginine-containing peptides have been explained by the increase of arginine concentration with the concomitant increase in endogenous NO levels (Udenigwe et al., 2012). These peptides also exhibited moderate *in vitro* inhibitory activity against ACE and a weak renin-inhibitory activity.

3.4. Identification of major peptides from proteinase K LFH <3 kDa and potential bioactivity of released peptides

Analysis of proteinase K LFH <3 kDa by HPLC–MS/MS allowed the identification of 37 peptides (Table 3). Proteinase K exhibits specificity for peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids (Ebeling et al., 1974). We have identified different peptides having at the C-terminus these residues such as alanine (A), valine (V), leucine (L), tyrosine (Y), phenylalanine (F) and tryptophan (W). The presence of some of these amino acids in ultimate position fulfil the rule proposed by Cheung, Wang, Ondetti, Sabo, and Cushman (1980) about residues being preferred for ACE inhibitors and substrates. These sequences could explain the *in vitro* ACE-inhibitory activity of proteinase K LFH <3 kDa (IC_{50} = 1.3 ± 0.1 μg/ml) although as we have shown here the hydrolyzate did not show inhibition of ACE-related vasoactive effects. Few LF-derived peptides with ACE-inhibitory and antihypertensive effects have been isolated from enzymatic digests. Peptide sequences RPYL [f(133–136)], LIWKL [f(266–270)], LNNSRAP [f(232–238)] and LRPVAA [f(74–79)] isolated from pepsin LFHs were shown as having antihypertensive properties (Lee, Cheng, Enomoto, & Nakamura, 2006; Ruiz-Giménez et al., 2012). None of these previous peptide sequences was identified in the present study. Only the peptide RPYL has been identified as part of the sequences GILRPY and GILRPYL found in the proteinase K LFH <3 kDa.

In contrast to ACE inhibitors, there is no information available about the relationship between amino acid sequence and ECE inhibitory effects. For the LfcinB-derived peptides with inhibitory effects on ECE-dependent vasoconstriction we could not establish these relationships taking into account *in vitro* and *ex vivo* data (Fernández-Musoles et al., 2010). None of these LfcinB-derived sequences was identified in the proteinase K LFH <3 kDa. With the aim of identifying those peptide sequences able to inhibit ECE, two of the most abundant peptides identified in the proteinase K LFH <3 kDa, fragments f(130–135) [GILRPY] and f(186–192) [REP-

Table 3
Identification of peptides contained in the proteinase K lactoferrin hydrolyzate with molecular mass lower than 3 kDa (LFH <3 kDa).

Observed mass[a]	Theoretical mass	Protein fragment	Identified sequence
785.5	785.47	f(28–35)	KLGAPSIT
1399.8	1399.76	f(66–77)	EAGRDPYKLRPV
1199.8	1199.68	f(68–77)	GRDPYKLRPV
983.6	983.48	f(83–91)	GTKESPQTH
825.5	825.40	f(85–91)	KESPQTH
877.6	877.50	f(97–104)	VVKKGSNF
717.5	717.42	f(130–135)	**GILRPY**[b]
830.6	830.50	f(130–136)	GILRPYL
930.5	930.42	f(186–192)	**REPYFGY**[b]
731.4	731.31	f(200–207)	QDGAGDVA
969.5	969.52	f(208–215)	FVKETTVF
799.5	799.41	f(216–222)	ENLPEKA
670.5	670.37	f(217–222)	NLPEKA
695.3	695.29	f(223–227)	DRDQY
824.4	824.33	f(223–228)	DRDQYE
937.5	937.41	f(223–229)	DRDQYEL
1050.6	1050.50	f(223–230)	DRDQYELL
443.4	443.29	f(255–258)	VVAR
1160.7	1160.57	f(259–268)	SVDGKEDLIW
1185.8	1185.61	f(290–300)	GSPPGQRDLLF
944.6	944.51	f(293–300)	PGQRDLLF
471.3	471.28	f(309–312)	RIPS
698.5	698.44	f(309–314)	RIPSKV
971.6	971.54	f(309–317)	RIPSKVDSA
759.5	759.42	f(329–334)	KNLRET
801.5	801.43	f(335–341)	AEEVKAR
718.4	718.33	f(443–448)	NEGLTW
643.4	643.34	f(468–473)	NIPMGL
654.4	654.33	f(492–498)	APGADPK
774.5	774.34	f(534–541)	AEDVGDVA
630.5	630.35	f(560–564)	WAKNL
728.5	728.42	f(578–583)	RKPVTE
799.6	799.46	f(578–584)	RKPVTEA
486.2	486.26	f(652–656)	GGRPT
778.5	778.36	f(652–658)	GGRPTYE
1070.5	1070.47	f(652–660)	GGRPTYEEY
873.5	873.38	f(657–663)	YEEYLGT

[a] Calculated monoisotopic mass.
[b] Chemically synthesized peptides are labelled in bold.

YFGY], were chemically synthesized and their *in vitro* ECE-inhibitory activity was tested. As summarized in Table 2, both peptides showed significant ECE-inhibitory effects at the three concentrations assayed, with a maximum inhibition of 52% and 40% for GILRPY and REPYFGY, respectively. Results at 30 μM are in the low range of inhibition described for LfcinB-derived peptides which showed significant ECE-inhibitory effects ranging from 19% to 86% (Fernández-Musoles et al., 2010). In endothelial cells, the presence of 1 mM lactokinin provoked a 29% reduction of ET-1 release (Maes et al., 2004). Whether only the two main sequences identified are responsible for the observed ECE inhibiting and antihypertensive effects of proteinase K LFH <3 kDa requires further characterization studies.

4. Conclusions

Bovine LF is a source of antihypertensive peptides which can be released by controlled enzymatic hydrolysis. We have shown that LFHs of molecular mass lower than 3 kDa exert antihypertensive effects in SHRs suggesting their potential application as constituents of functional foods in the treatment of hypertension. Moreover, these hydrolyzates do not show hypotensive effects in normotensive rats. Data reported here demonstrate that pepsin LFH is a dual vasopeptidase (ACE/ECE) inhibitor with antivasoconstrictor effects whereas ECE would be the functional target of proteinase K LFH. Future efforts will be directed to establish the long-term effects of LFHs on the arterial blood pressure in SHRs and clarify their *in vivo* ACE- and ECE-inhibitory effects.

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REVIEW

Antihypertensive peptides from food proteins: a review

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High blood pressure is considered as a significant health problem worldwide. In addition to numerous preventive and therapeutic drug treatments, important advances have been achieved in the identification of dietary compounds that may contribute to cardiovascular health. Among these compounds, peptides with antihypertensive properties received special attention in the past 15 years. Although milk proteins are still the main source of antihypertensive peptides, recently a remarkable increase has been noticed in the report of antihypertensive peptides released from other dietary sources. Most of these peptides have demonstrated their properties by *in vitro* assays. However, the evidence for their beneficial antihypertensive effects has to be based on animal experiments and clinical trials. This paper reviews the current data of the blood pressure-lowering activity of food-derived peptides demonstrated *in vivo* (animal models and humans). Other aspects, such as the mechanism of action and bioavailability of these peptides which play a key role in their antihypertensive effects are also summarized in this review.

1. Introduction

Many of the physiological functions in an organism are mediated by peptides. The human body synthesizes antimicrobial peptides like defensins and opioid endorphins; blood pressure is regulated by peptides such as angiotensin-II or bradykinin, and endogenous peptides are also involved in the immune response. Apart from these, humans ingest an average of 50 to 70 g of food protein per day. When these proteins are ingested they are hydrolysed into free amino acids and a high amount of different peptides, some of which can be absorbed and transported by the blood stream. It has been reported that some of these food derived peptides share structural motifs with endogenous peptides, that would allow dietary peptides to interact with body receptors, as it occurs for opioid peptides; to inhibit certain regulatory enzymes; or compete with bacteria or viruses for binding to body receptors, and others. These peptides are known as food derived bioactive peptides and are attracting increasing interest because of their variety and multifunctionality. Among these, food derived antihypertensive peptides are undoubtedly the group of bioactive peptides from which more information is available.[1–5] Much work has been done to evaluate the *in vitro* activity of peptides on the angiotensin-I-converting enzyme (ACE), which plays an important role in the regulation of blood pressure. However, the complete correspondence between both *in vitro* and *in vivo* effects has not been demonstrated in many of the published studies. Discrepancy between ACE inhibitory (ACEI) and antihypertensive activity of peptides can be due to their further degradation during gastrointestinal digestion, the impossibility to reach the target organ in the organism in a sufficient amount or because other mechanisms different than ACE inhibition may be involved. Therefore, this review is focused on those peptide sequences or food protein hydrolysates with *in vivo* blood pressure effects, demonstrated in spontaneously hypertensive rats (SHR), which is an accepted animal model to study human essential hypertension, or in human trials. In addition, although milk peptides are also revised, special focus is done on peptides from other sources: egg, meat, plants, and marine animal proteins. Finally, for the aspects where more evidence is needed, that is, clinical studies, the mechanism of action and bioavailability of peptides have also received particular attention in this review.

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2. Antihypertensive effect of food-derived peptides

2.1. Antihypertensive effect of peptides derived from milk proteins

Epidemiological studies suggest that milk and dairy product consumption is inversely related to the risk of hypertension. These products are rich in minerals (*e.g.* calcium, potassium) that could lower blood pressure,[6] but other dairy components, such as proteins and their degradation products have also been linked to the antihypertensive effect of milk and dairy foods. Most biologically active peptides generated from milk proteins have demonstrated ACEI activity. In the last two decades, antihypertensive effects of some of these peptides has been evaluated in SHR, and the peptide sequences, doses and maximum decrease of systolic blood pressure (SBP) are summarised in Table 1. The

Table 1 Antihypertensive activity in spontaneously hypertensive rats of peptides derived from milk proteins by enzymatic hydrolysis and fermentation

Obtention procedure	Enzyme/microorganism	Protein fragment	Peptide sequence	Dose (mg kg^{-1})	SBP decrease (mm Hg)[a]	Reference
Hydrolysis	Trypsin	α_{s1}-CN f(23–34)	FFVAPFPGVFGK	100.0	−34.0	7
		α_{s1}-CN f(194–199)	TTMPLW	100.0	−13.6	
		β-CN f(177–183)	AVPYPQR	100.0	−10.0	
		CMP f(106–112)	MAIPPKK	10.0	−28.0	121
	Pepsin	α_{s1}-CN f(90–94)	RYLGY	5.0	−25.0	8
		α_{s1}-CN f(143–149)	AYFYPEL	5.0	−20.0	
		α_{s2}-CN f(89–95)	YQKFPQY	5.0	−15.0	
		α_{s2}-CN f(203–208)	PYVRYL	3.0	−23.4	122
		Lfcin f(20–25)	RRWQWR	10.0	−16.7	123
		Lfcin f(22–23)	WQ	10.0	−11.4	
	Gastric and pancreatic enzymes	α-La f(50–53)	YGLF	0.1	−23.4	102
	Pepsin, chymotrypsin and trypsin	κ-CN f(22–24)	IAK	4.0	−20.7	9
		κ-CN f(61–66)	YAKPVA	6.0	−23.1	
		κ-CN f(76–86)	WQVLPNAVPAK	7.0	−18.4	
		κ-CN f(98–105)	HPHPHLSF	10.0	−15.7	
	Proteinase K	β-CN f(59–61)	VYP	8.0	−21.0	124
		β-CN f(59–64)	VYPFPG	8.0	−22.0	
		β-CN f(80–90)	TPVVVPPFLQP	8.0	−8.0	
		β-Lg f(78–80)	IPA	8.0	−31.0	
		BSA f(221–222)	FP	8.0	−27.0	
	Proteinase of *L. helveticus* CP790	α_{s1}-CN f(104–109)	YKVPQL	2.0	−13.0	10
		α_{s2}-CN f(189–192)	AMPKPW	2.0	−5.0	
		α_{s2}-CN f(190–197)	MKPWIQPK	2.0	−3.0	10
		α_{s2}-CN f(198–202)	TKVIP	2.0	−9.0	
		β-CN f(140–143)	LQSW	2.0	−2.0	
		β-CN f(169–174)	KVLPVP	2.0	−32.2	
		β-CN f(169–175)	KVLPVPQ	2.0	−31.5	
	Thermolysin	β-Lg f(58–61)	LQKW	10.0	−18.1	125
		β-Lg f(103–105)	LLF	10.0	−29.0	
	AS1.398 neutral protease	κ-CN f(15–18); κ-CN f(25–30)	DERF; RYPSYG	300.0[b]	n.d.[b]	126
	Flavourzyme + *S. thermophilus* and *L. bulgaricus*	κ-CN f(58–61)	YPYY	3.4	−15.9	17
	Prozyme 6 and mixture of lactic acid bacteria	α_{s1}-CN f(162–164) or β-Lg f(17–19)	GVW or GTW	3.3	−22.0	20
Fermentation	*L. helveticus* CPN4	α_{s1}-CN f(146–147)	YP	2.0	−32.1	10
	L. helveticus and *S. cerevisiae*	β-CN f(74–76)	IPP	0.3	−28.3	12,13
		β-CN f(84–86)	VPP	0.6	−32.1	
	E. faecalis	β-CN f(58–76)	LVYPFPGPIPNSL-PQNIPP	6.0	−14.9	18,19
		β-CN f(133–138)	LHLPLP	3.0	−25.3	
		β-CN f(133–139)	LHLPLPL	10.0	−7.7	
		β-CN f(134–138)	HLPLP	7.0	−23.5	
		β-CN f(197–206)	VLGPVRGPFP	10.0	−16.2	
		β-CN f(201–209)	VRGPFPIIV	10.0	−16.1	
	Caprine kefir	β-CN f(58–68)	LVYPFTGPIPN	10.0	−28.0	9
	Manchego cheese	α_{s1}-CN f(102–109)	KKYNVPQL	10.0	−11.5	9,25
	Gouda cheese	α_{s1}-CN f(1–9)	RPKHPIKHQ	6.1	−9.3	24
		β-CN f(60–68)	YPFPGPIPN	7.5	−7.0	
	Enzyme-modified cheese	β-CN f(102–104)	MAP	3.0	−17.0	127

[a] Systolic blood pressure. [b] Effect observed after administration of casein hydrolysate.

release of antihypertensive milk peptides is commonly achieved by two different approaches: milk protein hydrolysis and milk fermentation. The first strategy has been widely used to produce digests from caseinates, individual casein fractions, whey protein concentrates and isolates, and individual whey proteins, by using one, or a combination of food-grade enzymes. As shown in Table 1, a number of peptides with potent antihypertensive properties have been identified from caseins and whey proteins with gastric and pancreatic enzymes. One of the first peptides with a proven antihypertensive effect in SHR was identified in a tryptic casein hydrolysate, where several *in vivo* assays were performed by the administration of high doses of the hydrolysate or pure synthetic peptides.[7] One of these peptides, corresponded to an α_{s1}-casein-derived peptide, with sequence FFVAPFPGVFGK. The casein hydrolyzate containing this peptide has been patented and commercialized in an antihypertensive product named Peptide C12®. Recently, α_{s1}-casein has also been reported as a source of other two potent peptide fragments, RYLGY and AYFYPEL, which provoked a notable reduction of SBP of 25 and 20 mm Hg, respectively at 5 mg kg^{-1}.[8] Combined action of pepsin, chymotrypsin and trypsin was required to liberate peptide fragments from κ-casein with potent antihypertensive activity in SHR.[9] Peptides IAK, YAKPVA, and WQVLPNAVPAK

showed a clear decrease in both SBP and diastolic blood pressure (DBP) (Table 1). However, peptide HPHPHLSF caused a significant decrease in the DBP of the SHR, but this sequence did not modify the SBP of these animals. Hydrolysis with food-grade enzymes from microorganisms has also become a common strategy to liberate bioactive peptides. Active sequences have been identified in a casein hydrolysate with a proteinase of *Lactobacillus helveticus* CP790.[10] At a dose of 2 mg kg^{-1}, peptides KVLPVP and KVLPVPQ got a SBP reduction of 32.2 and 31.5 mm Hg, respectively.

The second approach exploits the proteolytic system of lactic acid bacteria to hydrolyze milk caseins during the manufacture of dairy products, such as fermented milk and cheese. Peptides and amino acids liberated during the fermentation process are used as nitrogen sources which are necessary for bacterial growth.[11] Also, these peptides can exert different biological functions. Tri-peptides VPP and IPP, produced from milk fermentation with a combination of *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, were the first peptides for which blood pressure lowering properties were reported.[12,13] A single oral administration of sour milk (5 ml kg^{-1} of body weight) containing 0.6 mg kg^{-1} VPP and 0.3 mg kg^{-1} IPP significantly decreased the SBP from 6 to 8 h after administration. Long-term administration of fermented milk or VPP and IPP has also been demonstrated to be effective in reducing SBP in SHR.[14–16] Different strains of lactic acid bacteria, such as *Lactobacillus helveticus* CPN4, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, have been also shown to provoke liberation of peptides with antihypertensive activity in SHR.[10,17] Two peptides, corresponding to β-CN fragments LVYPFPG-PIPNSLPQNIPP and LHLPLP, have been isolated in fermented milk with *Enterococcus faecalis* and their potent ACEI activity and antihypertensive effect in SHR, after acute and long-term administration have been proven.[18,19] The administration of 2 mg kg^{-1} of peptide LHLPLP resulted in a significant decrease of the SBP in SHR 4 h post-administration. The administration of 6 mg kg^{-1} of peptide LVYPFPGPIPNSLPQNIPP caused a slight, but also significant, decrease in SBP and DBP. Recently, hydrolysis with food-grade enzymes and successive fermentation of milk with one or more lactic acid bacteria strains is being used to liberate peptides with proven antihypertensive effect in animals.[17,20]

During the maturation of cheese, endogenous milk enzymes, added coagulants and microbial enzymes act on the major milk proteins that are degraded into a large number of peptides, some of them with proven antihypertensive activity. Although these peptides are not in sufficient amounts to warrant clinical trials, some of them have been synthesized and tested in the rat model. Tri-peptides VPP and IPP have been identified and quantified in different cheese varieties by Bütikofer and co-workers who found in some varieties physiologically relevant amounts.[21,22] Meyer and co-workers have reported that peptide concentration depends on cheese variety and other factors, indicating the importance of developing a reproducible cheese-making process with selected cultures to produce high concentrations of these peptides useful for being used in clinical trials.[23] Gouda and Manchego cheese are two other cheese varieties containing potent antihypertensive peptides derived from α_{s1}-casein and β-casein (Table 1).[9,24,25]

2.2. Antihypertensive effect of peptides derived from egg proteins

The egg, *per se*, is an important source of bioactive peptides and antihypertensive peptides specifically.[26–28] To date, several egg-derived peptides have been reported with a significant antihypertensive activity tested in SHR (Table 2). Over a decade ago, two egg-derived antihypertensive peptides were obtained from a hydrolysate of ovalbumin, the major egg white protein. Both proved to show antihypertensive activity in SHR with an associated vasorelaxing mechanism.[29] Ovokinin (FRADHPFL), resulting from the pepsin hydrolysate, had an important vasorelaxing activity in canine mesenteric arteries by bradykinin B_1 receptors.[30] Its antihypertensive effect in SHR, obtained at high doses (14.5 mm Hg at 100 mg kg^{-1} SHR), was greatly improved to a fourth part of dosage needed by applying it into emulsions with egg yolk, which leads to thinking that the phospholipids enhanced its absorption and protected it from intestinal peptidases.[31] The second egg-derived antihypertensive peptide, product of chymotrypsin ovalbumin hydrolysis, was characterized as ovokinin 2–7 (RADHPF) and had higher activity in SHR than ovokinin, getting the same effect with a ten times lower dosage. Furthermore, oral administration of ovokinin 2–7, did not lower the blood pressure of normotensive Wistar–Kyoto rats. Its mechanism of action was related to the nitric oxide (NO)-dependent vasorelaxation, observed in an isolated SHR mesenteric artery.[32] Later, when studying the ACEI activity of different ovalbumin hydrolysates, few peptides with significant ACEI activity were identified in a peptic hydrolysate. However, only LW presented a mild antihypertensive activity in SHR, getting a maximum reduction of 22 mm Hg after 2 h of 60 mg kg^{-1} SHR.[33] Consequently, these results again demonstrated the partial correspondence between *in vitro* and *in vivo* effects. Yoshii and co-workers studied a chicken egg yolk hydrolysate and got a dose-dependent antihypertensive activity (4–8%) in a long-term assay of 12 weeks, applying 20 to 500 mg kg^{-1} SHR. It was suggested that this effect could be related to an ACEI activity.[34]

Based on the criteria of seeking the highest *in vitro* ACEI activity, Miguel and co-workers obtained a pepsin egg white hydrolysate that exhibited a remarkable antihypertensive activity in short and long-term assays in SHR, as well as its three major ACEI peptides (YAEERYPIL, RADHPFL and IVF).[35,36] These peptides, at doses of 2, 2 and 4 mg kg^{-1} SHR, respectively, achieved a SBP reduction of around 30 mm Hg 6 h after oral administration. A further study with gastrointestinal simulation reported that these peptides, like the hydrolysate, maintained their antihypertensive activity but were degraded into fragments whose ACEI activity was notably reduced.[37] This result, together with additional studies of vasodilator and ACEI activities of the hydrolysate, peptides and digests, suggested that the antihypertensive action was exerted by a vasodilator mechanism independent of ACE inhibition.[38–40] In 2007, Miguel and co-workers identified two novel sequences derived from egg white proteins (YRGGLEPINF and ESIINF), that produced a significant reduction of approximately 30 mm Hg after oral administration of 10 mg kg^{-1} SHR. Similarly, a vascular-relaxing mechanism was attributed to explain the antihypertensive activity observed.[41]

Table 2 Antihypertensive activity in spontaneously hypertensive rats of peptides derived from egg proteins

Source	Administered product	SBP decrease (mm Hg)	Dose (mg kg^{-1})	Reference
Egg white hydrolysate	YRGGLEPINF	−30	10	41
	ESIINF	−30	10	
	Hydrolysate	−39.1	100	35
	YAEERYPIL	−31.6	2	35
	RADHPFL	−34.1	2	
	IVF	−31.7	4	
Ovalbumin hydrolysate	RADHPF + egg yolk	−10	10	32
	FRADHPFL + egg yolk	−17.6	25	31
	LW	−22	60	33
Egg yolk hydrolysate	Hydrolysate	−7	20	34

2.3. Antihypertensive effect of peptides derived from other animal proteins

Although milk and egg proteins are still the main source of antihypertensive peptides, recently a remarkable increase has been noticed in the number of reports of antihypertensive peptides released from other dietary sources, among which meat and marine animal products have been highlighted.[42,43] In addition, it should be noted that several antihypertensive peptides have been isolated from meat and marine by-products or wastes, a strategy that might provide substantial environmental and cost benefits.[44] Collagen and gelatin have attracted the attention of many researchers in the last decade, thanks to the optimization of extraction conditions. The most abundant sources of collagen and gelatin are porcine skin and bovine hide as well as bones, tendons and cartilages. However, by-products from non-mammalian species, such as poultry or fish, have received considerable attention in recent years.[45] Different food-derived protein sources of higher commercial value have also been under consideration for the collection of antihypertensive peptides, which have been successfully reported in both meat and marine animals.[29] In this context, examples of peptides and hydrolysates with a demonstrated *in vivo* antihypertensive activity from different sources have been described (Table 3).

The hydrolysates and peptides assayed *in vivo* have been generally obtained by enzymatic proteolysis, covering a wide range of potential enzymes, such as pepsin[46–48] or some less used enzymes as orientase.[49] Furthermore, it has been usual to apply consecutive treatments with different enzymes like bromelain and alcalase in sea cucumbers[50,51] or Protamex and Flavourzyme in freshwater clams.[52] Taking into account the studies in SHR, a wide variation has been observed in blood pressure response through the SBP measurements. Considering for each case the doses employed and the antihypertensive effect observed in both intensity and duration, examples with significant activities have been reported. A thermolysin hydrolysate of dried bonito, a traditional Japanese food, had special relevance among antihypertensive food-derived products.[53] Based on *in vivo* studies in SHR and clinical studies in borderline and mildly hypertensive subjects, an U. S. patent has been registered and implemented in a commercial product named Vasotensin® that ensures to support healthy blood pressure levels by supplying bioactive peptides from bonito fish. As an active component, the peptide LKPNM presented antihypertensive activity in SHR with a reduction of 10 mm Hg at 4 h after ingestion with a dose of 8 mg kg^{-1} SHR, as well as its hydrolysis product, the peptide LKP, getting similar effect but with a lower dose (4.5 mg kg^{-1} SHR) and earlier response (2 h after ingestion).[54]

Among pure peptides, potent antihypertensive sequences from meat proteins are described as MNPPK and ITTNP, which were released in the thermolysin hydrolysis of porcine muscle myosin. Both exhibited a maximum SBP reduction higher than 20 mm Hg at low dosage of 1 mg kg^{-1} SHR.[55] Regarding marine sources, LHP, from a hydrolysate of shrimp, showed a significant reduction of 16–18 mm Hg between 4 and 6 h after oral administration of 2 mg kg^{-1} SHR. When the dose was increased to 6 mg kg^{-1} SHR, the antihypertensive activity reached a maximum of 36 mm Hg at 6 h, getting a reduction in SBP from 2 to 8 h after ingestion.[56] Other peptides were found to be particularly active, such as AVF and VF, from an insect protein digestion,[57] YYRA from chicken bone hydrolysate[47] and KRVIQY from porcine myosin hydrolysate.[58] Longer peptides have been also reported with an important antihypertensive activity at 10 mg kg^{-1} SHR such as VKKVLGNP from another porcine myosin hydrolysate (24 mm Hg at 3 h),[59] GDLGKTTTVSNWSPPKYKDTP from tuna frame hydrolysate (21 mm Hg at 6 h),[48] as well as WPEAAELMMEVDP[60] and MIFPGAGGPEL[61] from tuna muscle and yellowfin sole frame hydrolysate, respectively, giving rise to a reduction of 20 mm Hg between 3–9 h.

2.4. Antihypertensive effect of peptides derived from plant proteins

Vegetable proteins have been less studied as a source of antihypertensive peptides than milk and other animal origin proteins but their relevance in this area is increasing. Among these, soybean proteins are receiving special attention as a source of bioactive peptides. As the major protein resource in many countries, soybean is consumed as tofu, soy milk, soy flour, soy protein isolate, tempeh and miso. Several sequences have been proposed as responsible for the antihypertensive activity of soy protein hydrolysates and fermented products (Table 4), but only the peptide HHL derived from fermented soy paste was assayed in pure form in SHR, where a decrease of 32 mm Hg of SBP was reached at a dose of 100 mg kg^{-1}. With the aim to produce this tri-peptide efficiently, a method for obtaining a large quantity of recombinant HHL has been developed.[62] Compared with animal-derived bioactive peptides, the inherently associated isoflavones in soybean-derived products may affect uniquely the

Table 3 Antihypertensive activity in spontaneously hypertensive rats of peptides derived from other animal proteins

Source	Administered product	SBP decrease (mm Hg)	Dose (mg kg^{-1})	Potential active sequence	Reference
Chicken muscle	IKW	−17	60		33
	LKP	−18	60		
Porcine troponin hydrolysate	KRQKYDI	−9.6	10		46
Porcine myosin hydrolysate	MNPPK	−23	1		55
	ITTNP	−21	1		
	VKKVLGNP	−24	10		59
	KRVIQY	−23	10		58
Chicken bone extract hydrolysate	YYRA	−20	10		47
Chicken leg collagen hydrolysate	GA(Hyp)GL(Hyp)GP	−18	4.5		128
Porcine skin collagen hydrolysate	GF(Hyp)GP	−20	10		129
Insect protein digest	AVF	−13	5		57
	VF	−19	5		
Bullfrog muscle protein hydrolysate	GAAELPCSADWW	−10	10		130
Shrimp hydrolysate	Hydrolysate	−18.3	100		131
	LHP	−18	2		56
Oyster protein hydrolysate	Hydrolysate	−30	100		113
	DLTDY	−15	8		
	DY	−20	10		
	Hydrolysate	−12	20	VVYPWTQRF	132
Sea cucumber hydrolysate	Hydrolysate	−73	40	Peptide (E, D, P, G and A)	50
	MEGAQEAQGD	−19	3 μM kg^{-1}		51
Yellowfin sole frame protein hydrolysate	MIFPGAGGPEL	−22	10		61
Tuna frame protein hydrolysate	GDLGKTTTVSNWSPPKYKDTP	−21	10		48
Tuna dark muscle hydrolysate	WPEAAELMMEVDP	−20	10		60
Tuna cooking juice hydrolysate	Hydrolysate	−23	500		49
	+ Lecithin	−34	500		
	+ Liposome	−39	500		
	+ Arabic gum	−36	500		
Dried bonito hydrolysate	LKPNM	−10	8		54
	LKP	−5	2.25		
	IWHHT	−26	60		33
	IVGRPRHQG	−14	60		
	IVGRPR	−17	60		
	IY	−19	60		
	IW	−22	60		
	IWH	−30	60		
	IKP	−20	60		
Sea bream scales hydrolysate	Hydrolysate	−20	300	GY, VY, GF, VIY	133
Salmon muscle protein hydrolysate	Hydrolysate	−28	500	WA, VW, WM, MW, IW, LW	134
Squid skin collagen hydrolysate	Hydrolysate	−40	200		135

activity found. Isoflavones are thought to possess a favorable effect in reducing cardiovascular risk factors as well as vascular function.[63] However, on the basis of *in vitro* results and literature review, Wu and Muir[64] have indicated that the contribution of isoflavones to a blood-pressure-lowering effect in soybean ACEI peptides may be negligible. Similarly, it has been reported that the reduction of hypertension of a fermented product from soy milk was contributed mainly by peptides of 800–900 Da but it could be also attributable to γ-aminobutyric acid (GABA).[65] Additional studies are required to determine the antihypertensive activity of soy peptides.

Other widely consumed plant foods such as wheat, maize, rice, pea, corn and apricot almond have shown antihypertensive effects when derived peptide products have been administered to SHR (Table 4). In order to isolate protein-rich fractions, operations such as cleaning, milling, sieving, extrudation and even autolysis at controlled pH and temperature of the seeds, grains or fruits are needed. The optimization of the extraction procedure is usually performed on the basis of the ACEI activity results. Subsequently, proteolysis with alcalase, thermolisin, α-chymotrypsin, subtilisin, neutrase, papain or other proteases has been carried out. As a result, a variety of assayed extracts can be found. Several peptide sequences have been identified as responsible of the bioactivity of these sources. For instance, the substantial antihypertensive activity of TGVY (40 mm Hg at 30 mg kg^{-1} SHR), identified in a rice protein hydrolysate, could in part count for the potent blood pressure-lowering effect reported of this hydrolysate.[66]

Other protein sources where antihypertensive activity has been found are mainly consumed in Asian countries as mung bean, which is a popular food in China or the edible tuber of *Apios Americana* and sake in Japan.[67–70] Five sake lee di-peptides showed remarkable antihypertensive properties (a decrease in SBP from 24 to 32 mm Hg at 100 mg kg^{-1}) both in young and old SHR but the effect of two of them did not last in the older animals. The authors indicated that the effect of ACEI peptides decreased due to tissue aging but the activity of some peptides could be maintained because of the contribution of other hypotensive mechanisms.[70]

Some vegetable proteins obtained from industrial by-products have been explored as a source of antihypertensive peptides. Thus, fibroin prepared from discarded silk thread and

Table 4 Antihypertensive activity in spontaneously hypertensive rats of peptides derived from plant and other food source proteins

Source	Administered product	IC_{50}	SBP decrease (mm Hg)	Dose (mg kg^{-1})	Potential active sequence	Reference
Soy protein	Soy protein hydrolysate with protease D3	180 μg ml^{-1}	−17.1	500	NWGPLV, PNNKPFQ, EDENNPFYLR, GGFIE, IPPGVPYWT	136
Soy protein	Oligopeptides from soy alcalase hydrolysate	—	−38	100	DLP, DG	137,138
Fermented soybean paste	HHL	2.2 μg ml^{-1}	−61	5 (triple i.v.)	HHL	139
Fermented soy milk	Whey fraction	2.89 μg ml^{-1}	−19	—	—	65
Fermented soybean seasoning	10% FSS	454 μg ml^{-1}	−20	10% FSS	VG, GY, SY, AY, AI, VP, AF, GT, AW	140
Wheat gluten	Wheat protein hydrolysate	340 μg ml^{-1}	−13.9	500	—	136
Wheat bran	Wheat bran peptides	0.14 mg ml^{-1}	−45	10	LQP, IQP, LRP, VY, IY, TF	141,142
Pea protein	Pea digest	0.16 mg ml^{-1}	−44.4	50 (i.v.)	—	143
Rice protein	Alcalase hydrolyzate	0.14 mg ml^{-1}	−25.6	600	TGVY	66
	TGVY	18.2 μM	−40	30		
Rice dregs	Rice dregs hydrolysate	—	−26	50	—	144
Corn	Corn oligopeptides	1.020 mg ml^{-1}	−45	450	AY	145
Apricot almond	Neutrase and N120P hydrolysate	0.138 mg ml^{-1}	−20.8	800	—	146
Mung bean	Alcalase hydrolysate	0.64 mg ml^{-1}	−30.8	600	—	67
Mung bean sprout	Raw sprout extract	—	−41	600	—	68
Sake	Hydrolysate of sake lee	—	−32	1000	HY, VY, RF, VW, YW	70
	Peptide fraction of sake	—	−21	1000		
	HY	26.1 μM	−32	100		
	VY	7.1 μM	−31	100		
	RF	93.0 μM	−24	100		
	VW	1.4 μM	−26	100		
	YW	10.5 μM	−28	100		
Rapeseed protein	Subtilisin hydrolyzate	0.16 mg ml^{-1}	−15.5	500	IY, RIY, VW, VWIS	73
	VW	1.6 μM	−10.8	7.5		
	VWIS	30 μM	−12.5	12.5		
	RIY	28 μM	−11.3	7.5		
	IY	3.7 μM	−9.8	7.5		
Silkworm fibroin	GVGY	35 μM 68 μM 15 μM	−33	44	GVGY GY	71
Silk fibroin	Silk fibroin hydrolysate	0.34 mg ml^{-1}	−62.7	1200	GY	72
Wakame (*Undaria pinnatifida*)	VY	35.2 μM	−17	10	VY, IY, FY, IW	75
	IY	6.1 μM	−21	1		
	FY	42.3 μM	−26	0.1		
	IW	1.5 μM	−14	1		
	YH	5.1 μM	−50	50	YH, KY, FY, IY	76
	KY	7.7 μM	−45			
	FY	3.7 μM	−46			
	IY	2.7 μM	−33			
Nori (*Porphyra yezoensis*)	Nori oligopeptide	—	−10	200	AKYSY	147
	AKYSY	—	−30	10		
Spirulina platensis	IQP	5.77 μM	−30	10	IQP	77,148
Pleurotus cornucopiae	Water extract	6 mg ml^{-1}	−50	600	RLPSEFDLSAFLRA RLSGQTIEVTSEYLFRH	78

hydrolyzed with alcalase, released two major active peptides showing ACE-inhibitory activity. The administration of one of them, GVGY, to SHR showed a blood pressure-depressing effect in a dose-dependant manner.[71] More recently, using a modified hydrolysis procedure, silk fibroin hydrolysate has shown potent antihypertensive effects after chronic oral administration, the activity being attributed to the di-peptide GY.[72] Rapeseed meal is a by-product of the oil removal process, and is comprised of approximately 40% protein. Subtilisin digestion of rapeseed protein exerted a long-lasting antihypertensive effect following oral administration to SHR. RIY, a peptide derived from napin, a major protein of rapeseed, has been demonstrated to be the main contributor to the activity although the isolated peptides IY, VW and VWIS have also lowered blood pressure in SHR.[73]

2.5. Antihypertensive effect of peptides derived from other sources

The exploration of marine organisms has revealed numerous bioactive compounds of proteinaceous nature.[74] Macroalgal content of protein varies significantly with species (3–47% w/w dry weight) and the protein levels fluctuate with season. Protein hydrolysates have shown mostly antioxidant activity but interesting reports of antihypertensive effects can be found (Table 4). Wakame (*Undaria pinnatifida*), the most widely eaten brown seaweed, is the source of four di-peptides that have shown antihypertensive effect after a single oral administration in SHR.[75] A hot water extract from the same seaweed produced several di-peptides, among which four sequences showed a potent antihypertensive effect.[76] The di-peptide IY was reported in both studies with decreases of SBP of 21 and 33 mm Hg at doses of 1 and 50 mg kg^{-1} SHR, respectively. Nori (*Porphyra yezoensis*) and Spirulina (*Spirulina platensis*) have been the source of short peptides showing antihypertensive effects in SHR. IQP, derived from the last one, has shown a significant regulation of the expression of major components of the renin-angiotensin system accompanying the blood pressure reduction.[77] Recently, two oligopeptides of 14 and 17 amino acids, respectively, have been identified as responsible of the antihypertensive action on SHR of the water extract of *Pleurotus cornucopiae*, a mushroom that grows well in the stumps of latifoliate trees.[78]

Many of the sequences in these sections are included in the BIOPEP database of proteins and bioactive peptides (www.uwm.edu.pl/biochemia). By using this database it is possible to investigate the potential activity of several food proteins by quantifying the occurrence frequency of bioactive fragments.

3. Antihypertensive effect in clinical studies

Evidence of the beneficial effects of antihypertensive peptides has to be based on clinical data. Most research has been especially focused in tri-peptides IPP and VPP.[79–81] Most of the studies performed until 2008 found that the oral administration of these tri-peptides included in different formulas, fermented milk, dried product, fruit juice, *etc.*, produces a significant decrease in SBP and DBP at doses between 3 and 52 mg day^{-1}. These results have been included in two *meta*-analyses performed by Pripp and Xu and co-workers.[82,83] These *meta*-analyses described decreases around 5.0 mm Hg for SBP, and 2.3 mm Hg for DBP. However, other recent clinical trials have reached controversial results, since they do not find effect on human blood pressure in Dutch and Danish subjects.[84,85] It has been proposed that genetics or dietary habits could explain these conflicting results. In a recent *meta*-analysis with a total of 18 trials, it was found a reduction of 3.73 mm Hg for SBP and 1.97 mm Hg for DBP but it was highlighted that the effect was more evident in Asian subjects that in Caucasian ones.[86] For certain antihypertensive drugs, it has been reported that a polymorphism found in humans can affect the clinical effectiveness, and similarly, these differences could be also affecting clinical trials of functional ingredients.[87]

These clinical trials have also been used to investigate further cardiovascular benefits of peptides and the mechanism of action in humans. Although ACEI has been postulated as the underlying mechanism of these lactotripeptides, results about the inhibition of this enzyme are not conclusive in humans. Several studies have shown that ACEI activity of IPP and VPP was not affected by the oral administration of the peptides.[16,84] In a recent study with 70 Caucasian subjects where a significantly reduction of blood pressure was found in hypertensive subjects stage 1 (but not in prehypertensive subjects), no differences were found in plasma renin activity, or angiotensin I and II.[88] Therefore, other mechanisms could be implicated in the observed blood pressure reduction. In a randomized double-blind clinical trial with Mediterranean volunteers, it has been found a positive effect on pulse wave velocity (a biomarker of vascular rigidity) and a mild but significant change in biomarkers of cardiac flow and cardiac contractility.[86] In a previous study, it was found that the intake of fermented milk containing these peptides may decrease sympathetic activity, leading to a diminished heart rate variability, heart rate and total peripheral resistance, although differences did not reach statistical significance.[85] It has also been reported that lactotripeptides produced a decrease in blood pressure during night-time sleep and it was proposed that a lower intake of salt may increase this effect on blood pressure.[89]

Other antihypertensive peptides and compounds derived from food sources have been tested in human assays. A thermolysin digest of dried bonito has demonstrated antihypertensive effects in a double-blind, randomized; cross-over study in 61 borderline and mildly hypertensive subjects.[53] Fermented milk with *Lactobacillus casei* and *Lactococcus lactis* that contained GABA reduced the SBP, but not the DBP, in a randomized, placebo-controlled trial with 39 mildly hypertensive patients after a period of 12 weeks.[90] A yogurt enriched with casein hydrolysate containing α_{s1}-casein antihypertensive peptides (RYLGY and AYFYPEL) was evaluated in 71 hypertensive subjects (divided in placebo and active substance groups) and 50 normotensive volunteers that received only active substance. After 6 weeks of consuming the yogurt containing the active ingredient, the hypertensive patients showed a change in their SBP of 12.5 mm Hg, while no significant changes in blood pressure were detected in both the placebo and the normotensive group.[91]

4. Mechanism of action

ACE inhibition is the main mechanism studied for peptides with proven antihypertensive effects. ACE is a constituent enzyme of the renin-angiotensin system that plays a crucial role in blood pressure regulation and fluid and electrolyte balance.[92] A number of *in vivo* studies performed in SHR and hypertensive human volunteers have demonstrated that several food-derived ACEI peptides significantly reduce blood pressure, either after intravenous or oral administration. However, additional mechanisms of action are being demonstrated.[93] In relation to this fact, antihypertensive peptides VPP and IPP are the most extensively studied peptides. In addition to their ACEI activity at micromolar concentrations,[12,13] these tri-peptides have been demonstrated to increase plasma renin activity and levels in SHR rats 14 weeks post-treatment.[15] Recently, in SHR rats fed fermented milk containing these peptides, it has been demonstrated a notable decrease of serum ACE activity.[94,95] These tri-peptides have been also shown to protect endothelial function of isolated mesenteric arteries of rats after 24 h incubation.[96] Similar results

have been reported in humans with mild hypertension, observing an improvement of their vascular endothelial dysfunction without changes of their systemic blood pressure after administration of a casein hydrolysate containing VPP and IPP.[97] These data suggest that VPP and IPP have direct beneficial effects on the vasculature. Aihara and co-workers have demonstrated that VPP attenuates phorbol 12-myristate 13-acetate (PMA)-stimulated adhesion of monocyte THP-1 cell line to activated human umbilical vein endothelial cells through, at least partially, inhibition of JNK phosphorylation.[98] Yamaguchi and co-workers studied, by DNA microarray microanalysis, the effect of a 5-day repeated administration of VPP and IPP on gene expression of SHR abdominal aorta, reporting a significant increase for the endothelial nitric oxide synthase (eNOS) gene and the connexin 40 gene, which are involved in blood pressure regulation.[99] Expression of these genes was restored in the aortic tissue after treatment with these tri-peptides, suggesting that VPP and IPP might act *in vivo* as ACE inhibitors in the aorta and also have preventive potential in cardiovascular function.[100,101]

Opioid receptors are present in the central nervous system and in peripheral tissues, where they are involved (*e.g.*, in the regulation of circulation and blood pressure). It has been demonstrated that these receptors are implicated in the antihypertensive effect of some food peptides. Nurminen and co-workers demonstrated that the antihypertensive effect of α-lactorphin, a tetra-peptide (YGLF) formed by *in vitro* proteolysis of α-lactalbumin with pepsin and trypsin, was mediated through the vasodilatory action of binding to opioid receptors.[102] This peptide has been found to lower blood pressure in SHR and produce an endothelium-dependent relaxation of their mesenteric arteries that is inhibited by an eNOS inhibitor.[15] Therefore, a mechanism of action driven by the stimulation of peripheral opioid receptors and subsequent NO release causing vasodilatation has been proposed for this peptide. Release of other vasodilator substances like prostaglandin I_2 or carbon monoxide could also be implied in the blood-pressure-lowering effects of ACEI and antihypertensive peptides.[30,32,103] As an example, peptide lactokinin, which sequence is ALPMHIR, inhibits the release of an endothelial factor (ET-1) that evokes contractions in smooth muscle cells through mechanisms both dependent and independent of ACE inhibition.[104]

Oxidative stress is another significant causative factor for the initiation or progression of hypertension and several vascular diseases. It has been demonstrated that production of reactive oxygen species, such as superoxide anion and hydrogen peroxide, is increased in hypertensive subjects, in which NO synthesis and bioavailability of antioxidants are reduced.[105] Furthermore, antioxidant-rich diets have been shown to reduce blood pressure in SHR[106] and hypertensive humans.[107] Therefore, food-derived peptides with antioxidant properties might also have effect on blood pressure modulation. A dual (ACEI and antioxidant) activity has been demonstrated for different milk-derived peptides, being responsible for their antihypertensive effect proven in animals.[8]

In the last years, in-depth studies are being performed to identify new mechanisms of action of antihypertensive peptides. Yamada and co-workers have demonstrated that the peptide rapakinin relaxes the mesenteric artery of SHR *via* the prostaglandin I_2-IP receptor followed by CCK-CCK1 receptor pathway.[108] These authors have demonstrated that the antihypertensive activity of this peptide is induced mainly by CCK1 and IP-receptor-dependent vasorelaxation. Later on, cardiovascular benefits of a product based on a casein hydrolysate administered chronically (800 mg kg^{-1}) during 6 weeks to SHR have been reported. This product, containing the antihypertensive peptides RYLGY and AYFYPEL produces an attenuated development of hypertension, improves aorta and mesenteric acetylcholine relaxations and decreases left ventricular hypertrophy, accompanied by a significant decrease in interstitial fibrosis.[109] Two enzymes, whose inhibition is being suggested as the best alternative in hypertension prevention, are renin and platelet-activating factor acetylhydrolase (PAF-AH). Renin is an enzyme recognized as the initial and rate-limiting compound of the renin–angiotensin system. The first direct renin inhibitor, known as aliskiren, is currently under phase III trials to evaluate its use as an antihypertensive drug.[110] Three di-peptides liberated after pea protein hydrolysis with alcalase have been characterized as potent renin inhibitors.[111] PAF-AH is a circulating enzyme secreted by inflammatory cells and involved in atherosclerosis. The discovery of natural PAF-AH inhibitors and their incorporation into functional foods with activity against hypertension and cardiovascular diseases holds considerable potential.[112]

5. Bioavailability

One of the main difficulties in establishing a direct correspondence between *in vitro* and *in vivo* activities of bioactive peptides is the bioavailability. The physiological effect of bioactive peptides is determined by their capacity to reach the target organ in an active form. Oral administration of a bioactive peptide comprises a series of processes that must be considered on the final desired activity. The steps include the attack of gastrointestinal enzymes and brush border peptidases, absorption through the intestinal barrier with the possibility of active intracellular peptidases in the transcellular transport, as well as the blood enzymes, once they have reached the circulation.[3] This situation makes it highly unlikely that antihypertensive reported sequences are not subjected to any alteration before eliciting their activity. This concern is reflected on motivation of researches in knowing different aspects of the antihypertensive sequences bioavailability. One of the most studied is the effect that digestion has on antihypertensive peptides, for which purpose gastrointestinal simulations are implemented with different enzymes. For instance, protease digestion analysis of the antihypertensive peptide DLTDY from an oyster hydrolysate, revealed its vulnerability to the formation of shorter sequences. Among them, DY produced an important reduction of SBP and was suggested as final responsible for the antihypertensive activity of both penta-peptide and hydrolysate.[113] Another common aim is the study of intestinal absorption, for which *in vitro* tests are performed with the monolayer of intestinal cell lines, simulating intestinal epithelium, as well as the analysis of peptides and derivatives in blood samples after *in vivo* and clinical studies. This is the case of milk-derived LHLPLP that resisted gastrointestinal simulation but was degraded to HLPLP by cellular peptidases before crossing Caco-2 cell monolayer.[114,115] This sequence, HLPLP, has been identified in human

plasma after oral administration, which demonstrates its intestinal absorption in humans.[116]

By associating antihypertensive activity and the ACEI mechanism, a classification is established, taking into account the bioavailability factor.[33] Based on a preincubation with ACE, three groups are described: the inhibitor type, peptides whose IC_{50} value is not affected; the substrate type, peptides that are hydrolyzed by ACE to give peptides with weaker activity and prodrug-type inhibitors, peptides that are converted to true inhibitors by ACE or gastrointestinal proteases. Pro-drug inhibitory peptides are characterized by a delayed antihypertensive effect because it is understood that obtaining active form takes time.[51,58] A clear example is found in the peptide LKPNM, whose digested fragment LKP showed greater ACE inhibitory and antihypertensive activity, in addition to appearing faster.[54] The generation of active forms after digestion is not only attributable to the ACE inhibition mechanism but it has also been reported in other ones like the vasorelaxation related to several egg-derived peptides.[37,39]

The improvement of antihypertensive peptides bioavailability is conceived as an important goal when evaluating their effectiveness, thus its optimization has been expressed in many studies. For example, Ko and co-workers applied lecithin and egg yolk emulsifications, arabic gum microencapsulation and lipophilization to enhance the antihypertensive activity of a hydrolysate of tuna cooking juice. Among these treatments, lipophilization was the most effective, followed by microencapsulation and lecithin emulsification, getting for each of them a stronger effect than the obtained with the double untreated dosage.[49] According to the authors, these enhancing effects may be brought about by the changes in cell membrane permeation or the protection of oligopeptides. However, the egg yolk emulsification did not involve any remarkable advantage regarding the hydrolysate antihypertensive activity, conclusion that contrasts with the results obtained by Fujita and co-workers, where the antihypertensive effect of ovokinin (FRADHPFL) increased to the equivalent of four-times the untreated dosage after administration with egg yolk.[31] In this case, phospholipids were identified as responsible for enhancing the antihypertensive effect, particularly phosphatidylcholine, that could improve intestinal absorption or by protecting ovokinin of peptidases. Such differences indicate that there is not a single strategy in improving antihypertensive peptides bioavailability but each particular case must be studied due to the number of processes involved. Currently, many possibilities are reported for improving bioavailability,[117,118] such as development of peptide analogues that keep their biological activity,[108,119] stabilization with a wide nature of molecules to help transport through the intestinal barrier or provide resistance against enzymatic attacks, as well as microencapsulation that controls the release of functional peptides.[45]

6. Antihypertensive peptides in commercial products

An increasing number of foods sold in developed countries bears nutrition and health claims. In fact, it has been reported that daily consumption of a moderate amount of antihypertensive peptides could elicit a blood pressure reduction not far from that of synthetic drugs.[120] There are numerous available patents of products containing antihypertensive bioactive peptides. Table 5 lists a selection of registered products that are already in the market. Some of these products are under development by companies that are intended to exploit the hypotensive potential of the peptides. First, it is necessary to identify and quantify the active sequences. Antihypertensive peptides are only minor constituents in highly complex food matrices and, therefore, a monitoring of the large-scale production by hydrolytic or fermentative industrial process is mandatory. Second, extensive investigations to prove the antihypertensive effect in humans as well as the minimal dose to show this effect are necessary to fulfill the requirements of the legislation concerning functional foods. Developed countries have adopted regulations on this area during the last two decades, being Japan the pioneer in 1991 with the Foods for Special Health Use (FOSHU) legislation. Europe adopted a joint Regulation on Nutrition and Health Claims made on Foods in 2006 being the European Food Safety Authority (EFSA) the governmental agent responsible for verifying the scientific substantiation of the submitted claims, although the decision whether to authorise them corresponds to the European Commission and Member States. Inevitably, the steps necessary to achieve the commercialisation of these products represent a very demanding task.

7. General conclusions

Much work has been done with dietary antihypertensive peptides and evidence of their effect has been built in animal and clinical studies. However, certain aspects, such as identification of the active form in the organism and the different mechanisms of action that contribute in the antihypertensive effect still need to be further investigated. In this regard, the use of new technologies like DNA microarray technology to study the effect of peptides on gene expression can offer new insights in the mode of action of these molecules.[99] In any case, evidence to support health claims have to be based on human clinical trials.

Together with the development of new functional ingredients, the effect of protein-containing food that is usually consumed merits to be considered. Food can be a natural source of bioactive peptides due to hydrolytic processes that occurs during gastrointestinal digestion. In addition, there is growing evidence that the peptide sequences released during digestion can vary depending on the manufacture process, food composition or previous proteolytic procedures, such as fermentation. Although there is still poor knowledge on the resistance of peptides to gastric degradation, unexpected results on the cleavage of specific peptide bonds have been reported. This reinforces the need to perform *in vivo* digestion experiments, in order to know the susceptibility of peptides to gastrointestinal enzymes hydrolysis in a given food matrix.

It has also been highlighted that there is a huge potential for obtaining antihypertensive peptides from protein sources other than milk. Many *in vivo* active sequences come from plant or animal muscle proteins. These findings open an interesting field aiming the revalorisation of an important amount of by-products, especially from the meat and seafood processing industry.

Table 5 Commercial food products containing peptides with proven antihypertensive activity

Name	Product type	Publication number [Reference]	Active sequence(s)
Calpis®	Fermented milk product	EP0323283 [149]	VPP, IPP
Vasotensin®	Bonito protein hydrolyzate	US5314807 [150]	LKPNM, LKP
Peptide C12®	Casein hydrolyzate	JP62270533 [151]	FFVAPFPEVFGK
Evolus®	Fermented milk product	US6972282 [152]	VPP, IPP
Tensiocontrol®	Egg protein hydrolyzate	WO2005012355 [153]	RADHPFL, YAEERYPIL, IVF
Biozate®	Whey protein hydrolyzate	US6998259 [154]	Whey peptides
Lowpept®	Casein hydrolyzate	WO2005012355 [122]	RYLGY, AYFYPEL

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3

Peptides

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CONTENTS

3.1 Introduction

Over the last decade many efforts have been made to develop techniques and methods for the separation, purification, and characterization of food proteins and peptides (Careri et al., 2003). The qualitative and **Q1** quantitative analysis of peptides by different high-performance liquid chromatography (HPLC) modes has become a common practice in most laboratories due of its versatility, short analysis times, high resolution, and effective separations, and because its suitability to automation procedure (González de Llano

Q2 et al., 2003). Practically all known mechanisms have been employed in the chromatographic separations of food peptides, for example, separation based on molecular size (size-exclusion chromatography, SEC), on charge (ion-exchange chromatography, IEX), on hydrophilic interaction (hydrophilic interaction liquid chromatography, HILIC), on affinity (affinity chromatography, AC), and even on combinations thereof of these (Issaq, 2001). However, reversed-phase (RP) chromatography is the most commonly used technique to separate mixtures of peptides from foodstuffs (Polo et al., 2000). In addition, mass spectrometry (MS) detection has become the preferred choice for peptide analysis in foodstuff and protein hydrolysates in laboratories dealing with proteome research and food analysis.

Peptides are defined as biological molecules that share the common characteristic of being made up of chains of amino acids joined together by peptide bonds. Therefore, peptides are formed of two or more amino acids when the carboxyl group of one amino acid reacts with the amino group of another amino acid, resulting in an amide (peptide) bond. The variety of existing amino acids and the large number of possible combinations to form peptides makes this group of compounds very heterogeneous.

Peptides are widely distributed in nature and found in many different organisms from vegetal and animal sources. Peptides and proteins contribute to physical properties, biological activities, and sensory characteristics of foods. The breakdown of proteins to smaller peptides and free amino acids during proteolysis influence the quality of fermented or otherwise ripened dairy products, fish, meat, cereals, and vegetables (Pripp et al., 2005). Functional applications include improved whipping, gelling, and solubility of the formulated products. In addition, peptides are attributed with a number of different biological properties, including antimicrobial activity, blood pressure-lowering (angiotensin-converting enzyme (ACE) inhibitory activity), cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of mineral absorption and/or bioavailability, cyto- or immunomodulatory effects, and opiod activities (Korhonen and Pihlanto, 2003; Yamamoto et al., 2003; Hartmann and Meisel, 2007; Hernández-Ledesma et al., 2008; López-Expósito and Recio, 2008). Peptides have been also investigated for their sensorial properties, which are known to determine sweet and bitter taste in foodstuffs (Kilara
Q3 and Panyam, 2003; Gonzalez de Llano et al., 2004).

It is important to characterize food peptides on the basis of their size, sequence, posttranslational (e.g., phosphorylation, glycosylation), and chemical modifications occurring during processing, storage, and so on. This implies separation, identification, and quantification of peptides formed as a part of complex mixtures. This information can be used to explain their influence on the biological activity, flavor, and functional properties of food, and can also be used for product authenticity and history assessment (Recio and López-Fandiño, 2009). Peptide analysis can also be applied for the characterization of food proteins using a proteomic approach or to assess the specificity and suitability of proteolytic enzymes (Minkiewicz et al., 2008).

Chemical and physical properties of peptides such as length, sequence, net charge, nearest neighbor, degree of hydrophobicity, solubility, and polypeptide structure determine, among other properties, its solubility and its retention in normal-phase and reversed phase, as well as IEX and SEC (Issaq et al., 2009). Solubility is an important parameter in peptide separation by HPLC that influences the selection of the mobile phase. Table 3.1 shows solubility guidelines based on information taken from Issaq et al. (2009).

This chapter describes the use of HPLC techniques for the analysis of food peptides. A first part is dedicated to sample preparation, followed by a description of the main separation modes used for peptide analysis and, finally, an overview of the most used detection techniques. The second part provides information of the different applications of HPLC analysis of food peptides. Particular attention is devoted to food peptidomics and the characterization of biologically active peptides, the evaluation of their sensory and functional properties, and the study of peptide allergenecity, among other applications.

3.2 Food Peptide Analysis

3.2.1 Sample Preparation

The PERFECT situation, for each analyst, is to have a method that enables a direct analysis without the need for any additional steps. Unfortunately, such a situation happens very rarely in the HPLC analysis

TABLE 3.1

Influence of the Chemical Properties of the Different Amino Acids on Peptide Solubility in the HPLC Mobile Phase

Types of Peptides	Solubility in Phase Mobile	Observations
Peptides shorter than 5 residues	Aqueous media	Except when all the residues are very hydrophobic (Trp, Ile, Leu, Phe, Met, Val or Tyr)
Hydrophilic peptides containing >25% charged residues (Glu, Asp, Lys, Arg, and His) and <25% hydrophobic residues	Aqueous media	Both acidic peptides (Glu + Asp residues < Lys + Arg + His residues) and basic peptides (Lys + Arg + His residues > Glu + Asp residues) are more soluble at neutral pH than at acidic pH
Hydrophobic peptides containing 50–75% hydrophobic residues	Insoluble or only partially soluble in aqueous media	Initial solubilization in strong solvents (i.e., dimethyl formamide, ACN, isopropyl alcohol, ethanol, acetic acid, 4–8 M guanidine hydrochloride or urea, dimethyl sulfoxide)
Very hydrophobic peptides containing >75% hydrophobic residues	Insoluble in aqueous media	Initial solubilization in very strong solvents (i.e., TFA or formic acid)
Peptides containing >75% proportion of Ser, Thr, Glu, Asp, Lys, Arg, His, Asn, Gln, or Tyr	Tendency to form gels in concentrated aqueous solutions	Initial solubilization in strong solvents (i.e., dimethyl formamide, ACN, isopropyl alcohol, ethanol, acetic acid, 4–8 M guanidine hydrochloride or urea, dimethyl sulfoxide)

of peptides from foods and it is limited to only some cases (Cheison et al., 2010). Mostly, food samples contain a low concentration of peptides as well as a large variety of nonpeptidic constituents (i.e., lipids, sugars) that can interfere with peptide analysis. Hence, even taking into account the last analytical advances, it is usually necessary that the application of pretreatment techniques that involve, on the one hand, peptide concentration for suitable detection and, on the other, the elimination of interfering components (González de Llano et al., 2004; Gilani et al., 2008; Asensio-Ramos et al., 2009; Poliwoda and Wieczorek, 2009). Several options may be taken as it is summarized in Table 3.2. In general, food samples are first subjected to a preliminary sample cleanup step to remove interferents, and then separation and purification steps are applied.

3.2.1.1 Extraction and Preliminary Sample Clean-Up

Extraction is an essential procedure when analyzing solid samples. In general, hydrophilic peptides are extracted with homogenization in water, solutions of organic acids or sodium chloride, whereas for highly hydrophobic peptides mixtures of organic solvents are used (i.e., methanol and chloroform). Regarding food samples, homogenization in water has been mainly applied on cheese samples. Mostly, the ratio of water to cheese was 2:1 in homogenization process, followed by an incubation step of an hour at 60°C (Taborda et al., 2007; Meyer et al., 2009).

Peptide extraction is usually followed by a deproteinization step. In food samples, deproteinization is carried out by precipitation of protein using several agents. After precipitation, centrifugation, filtration or both methods are used to separate proteins from soluble peptides. The selectivity of precipitation directly depends on the type of precipitating agent applied. Most typical precipitation agents are solutions containing organic solvents such as ethanol, methanol, acetone or acids solutions with trichloroacetic acid or trifluoroacetic acid. Salting-out precipitation, based on polarity, with high concentration of salts like ammonium sulfate or precipitation by adjusting the pH to the isoelectric point of protein (Contreras et al., 2010; Pihlanto et al., 2010) are other options. In addition, it could be considered regarding the application of heat treatments to denature the protein or a ultracentrifugation step at high speed to eliminate the protein (Gómez-Ruiz et al., 2007; Ho et al., 2010). Deproteinization also can act as fractionation procedure because peptide solubility depends on precipitant agent and its proportion. Cheng et al. (2010a,b) fractionated a potato protein hydrolysate on the basis of its polarity using ammonium sulfate precipitation, which

TABLE 3.2

Examples of Sample Preparation for Peptide Analysis by HPLC

Food Matrix	Analyte	Extraction and Clean-Up	Ultrafiltration [MW Cut-off]	Low-Pressure Chromatography	SPE	Additional Treatment	References
Dairy Products							
Dry-off cows milk	Peptide profile	Centrifugation and ultracentrifugation	10 kDa (centrifugal filter)	—	—	—	(Ho et al., 2010)
Fermented milk	Antioxidant peptides	Centrifugation	—	—	Sep-pak C-18	Preparative RP-HPLC	(Hernández-Ledesma et al., 2005)
Manchego cheese	Sensory peptides	Homogenization, centrifugation, filtration and ultracentrifugation	1 kDa (membrane)	SEC (Sephadex G-10)	—	—	(Taborda et al., 2007; Gómez-Ruiz et al. 2007)
Cheddar cheese	ACE inhibitory peptides	Homogenization, centrifugation and filtration	—	—	—	Preparative RP-HPLC	(Ong and Shah, 2008)
Ovine α_{s2}–casein digest	Antibacterial peptides	Centrifugation	—	IEX (Sepharose SP)	—	Preparative RP-HPLC	(López-Expósito et al., 2006)
Whey protein concentrate hydrolysate	Antioxidant peptides	Centrifugation and filtration	3 kDa (membranes)	—	—	—	(Contreras et al., 2011)
Yak milk casein hydrolysate	ACE inhibitory peptides	pH adjustment and centrifugation	10 and 6 kDa (membranes)	SEC (Sephadex G-25)	—	Preparative RP-HPLC	(Jiang et al., 2007)
Fish and Meat							
Cuttlefish protein hydrolysate	ACE inhibitory peptides	Centrifugation	—	SEC (Sephadex G-25)	—	—	(Balti et al., 2010)
Loach protein hydrolysate	Antioxidant peptides	Centrifugation	10, 3, and 0.5 kDa (membranes)	IEX (Cation-exchange) SEC (Sephadex G-25)	—	Preparative RP-HPLC	(You et al., 2010)
Hake protein hydrolysate	Antioxidant and ACE inhibitory peptides	Centrifugation, filtration and freeze-died samples homogenization	10, 3, and 1 kDa (membranes)	—	—	Preparative RP-HPLC	(Samaranayaka et al., 2010)
Pollack frame protein hydrolysate	Antioxidant peptides	—	30, 10, 5, 3, and 1 kDa (membranes)	IEX (SP-Sephadex C-25) SEC (Sephadex G-25)	—	Preparative RP-HPLC	(Je et al., 2005)

Dry-cured ham	Peptide profile	Homogenization, centrifugation, filtration and deproteinization by ethanol addition	—	SEC (Sephadex G-25)	—	Preparative RP-HPLC	(Mora et al., 2010, 2011)
Bovine liver protein hydrolysate	Antioxidant peptides	Centrifugation	10 and 3 kDa (membranes)	—	—	Preparative RP-HPLC	(Di Bernardini et al., 2011)
Porcine plasma protein hydrolysate	Antioxidant peptides	—	10, 6, and 3 kDa (membranes)	IEX (Sepharose SP)	—	Preparative RP-HPLC	(Liu et al., 2010b)
Pork meat digest	Peptide profile	Centrifugation and deproteinization by ethanol addition	—	—	—	—	(Escudero et al., 2010b)
Eggs							
Egg white protein hydrolysate	ACE inhibitory peptides	Centrifugation	—	SEC (Sephadex G-25) IEX (Sephadex C-25)	—	—	(Liu et al., 2010a)
Egg white protein hydrolysate	ACE inhibitory peptides	Centrifugation	3 kDa (centrifugal filter)	—	—	Preparative RP-HPLC	(Miguel et al., 2004)
Drinks							
White and red wines	ACE inhibitory peptides	Centrifugation	10 kDa (centrifugal filter)	SEC (Sephadex LH-20)	—	—	(Pozo-Bayón et al., 2007)
Champagne wine	Peptide profile	—	1 kDa (membrane)	—	—	—	(Person et al., 2004)
Vegetable Foods							
Buckwheat protein digest	Antioxidant peptides	Filtration	—	SEC (Sephadex G-25)	—	—	(Ma et al., 2010)
Corn zein hydrolysate	Antioxidant peptides	Pigments extraction and centrifugation	10, 5, 3, and 1 kDa (membranes)	—	—	Preparative RP-HPLC	(Tang et al., 2010)
Fermented soybean extract	ACE inhibitory peptides	Filtration and dialysis	30, 10 and 3 kDa (membranes)	IEX (Sepharose SP)	—	Preparative SEC and RP-HPLC	(Rho et al., 2009)
Soy protein hydrolysate product	Peptide molecular mass distribution	pH adjustment and centrifugation	—	—	—	—	(Johns et al., 2011)

continued

TABLE 3.2 (continued)

Examples of Sample Preparation for Peptide Analysis by HPLC

Food Matrix	Analyte	Extraction and Clean-Up	Ultrafiltration [MW Cut-off]	Low-Pressure Chromatography	SPE	Additional Treatment	References
Pea protein hydrolysate	Inhibitory peptides	Centrifugation	1 kDa (membrane)	—	Bond Elut SCX (cation exchange)	—	(Li and Aluko, 2010a)
Potato protein hydrolysate	Antioxidant peptides	Salting-out precipitation and centrifugation	—	SEC (Sephadex G-25)	—	Preparative RP-HPLC	(Cheng et al., 2010a,b)
Japanese soy sauce	Sensory peptides	Filtration	10, 3, and 0.5 kDa (membranes)	SEC (Sephadex G-25) SEC (Sephadex G-10)	—	—	(Lioe et al., 2006)

Note: ACE: angiotensin I-converting enzyme, IEX: ion exchange chromatography, MW: molecular weight, RP: reverse phase, SEC: size exclusion chromatography.

was gradually added to the hydrolysate to reach 30% (w/v) saturation followed by centrifugation. Subsequently, the concentration of ammonium sulfate in the supernatant was increased in a series from 30% to 50%, from 50% to 70%, and from 70% to 90% saturation, which yielded three additional precipitates, respectively.

In some cases, the application of homogenization and/or deproteinization is enough to proceed to peptide analysis (Contreras et al., 2010). Unfortunately, mostly treated samples need additional steps to achieve a suitable isolation and concentration of peptides.

3.2.1.2 Fractionation by Ultrafiltration

In food samples, ultrafiltration is mainly useful for fractionating peptides in function of their molecular size, as well as to remove proteins and other macromolecules. Mostly, membranes are made of polysulfone or cellulose derivatives. Cellulose membranes have excellent hydrophilicity, which is very important in minimizing fouling, but they possess low chemical resistance and poor mechanical strength. However, polysulfone membranes provide high rigidity but fouled earlier because of their hydrophobicity (Doyen et al., 2011). Commercially, membranes offer a wide range of cut-offs (500 Da to 100 kDa) and different formats like centrifugal units or cassettes for peristaltic lab systems. Fractionation of peptides has been achieved in food samples by applying ultrafiltration with more than one cut-off membrane. For instance, Di Bernardini et al. (2011) fractionated soluble peptides of a hydrolysate of bovine liver protein in two molecular weight ranges by ultrafiltration of the hydrolysate supernatants with 10 kDa and 3 kDa cut-off membranes. Tang et al. (2010) also applied an ultrafiltration system with 10, 5, 3, and 1 kDa cutoff membranes to obtain peptide fractions of different molecular weight from a zein hydrolysate. Regarding food bioactive peptides, like ACE inhibitory peptides, considering the size of most of them, ultrafiltration with a cut-off of 3 or 5 kDa seems a good choice, whereas with a 1 kDa cut-off membrane some of the active peptides may be lost (Lopez-Fandiño et al., 2006). **Q3**

In summary, ultrafiltration presents some advantages such as sample is not diluted or organic solvents are not required. Therefore, in some cases after ultrafiltration step, no additional fractionation processes are applied before HPLC analysis, like in cheese (Bütikofer et al., 2008) or drink samples (Person et al., 2004). Nevertheless, samples often need further pretreatment procedures that improved mainly the analyte concentration.

3.2.1.3 Fractionation by Low-Pressure Liquid Chromatography

Application of low-pressure liquid chromatographic methods in food samples is mainly used for peptide fractionation. Nevertheless, in some case, the aim is to remove salts or other endogenous compounds that may interfere in peptides analysis (Cheng et al., 2010a,b).

Low-pressure SEC fractionates peptides on basis of their molecular size. For this purpose, several resins with different pore sizes are commercially available. Cross-linked dextran (Sephadex) resins are mostly used but polyacrylamide (BioGel P) or divinylbenzene polymers are offered as stationary phases (Poliwoda and Wieczorek, 2009). Depending on resin composition, peptides are eluted with water, organic acids, ammonia or ammonium salts, even as alcoholic solutions that reduce potential hydrophobic interactions. For instance, in Sephadex columns, peptides are eluted with Milli-Q or distilled water. Several examples of SEC in food peptide fractionation have been recently reported: Lee et al. (2011) applied SEC to fractionate peptides from a skate skin hydrolysate to study their ACE inhibitory activity, Pozo-Bayón et al. (2007) in white and red wines to identify also ACE inhibitory peptides, and Ma et al. (2010) to study radical-scavenging peptides from a buckwheat protein digest.

Low-pressure IEX constitutes another technique for peptide fractionation in food analysis. In this case, peptides are fractionated according to their net surface charge/polarity. Porous or nonporous matrices with hydrophilic materials such as cellulose, cross-linked dextrans, polystyrene polymers (Dowex resins) or Bio-Rex membranes are very useful as anion or cation exchange stationary phases. These matrices are substituted with functional groups that determine the charge of the medium (e.g., quaternary ammonium, diethylaminoethyl, sulfopropyl, carboxymethyl, etc.). Peptide elution is achieved by using gradients of increasing ionic strength at constant pH (Liu et al. 2010b), changing the pH of mobile

phase (López-Expósito et al., 2006) or applying buffers containing pyridine to eliminate hydrophobic interactions (González de Llano et al., 2004).

Off-line combination of IEX and SEC has been reported in some food peptide analysis. A representative example is found in the work of Liu et al. (2010a), who fractionated an egg white protein hydrolysate by SEC with Sephadex G-25 resin followed by IEX with a Sephadex C-25 column of those fractions with the highest ACE inhibitory activity. Similar fractionation strategy has been also used in the study of antioxidant peptides in a fish protein hydrolysate (You et al., 2010) and for the evaluation of the peptide contribution to umami taste of soy sauces (Lioe et al., 2006).

3.2.1.4 Fractionation by Solid-Phase Extraction

Solid-phase extraction (SPE) is a pretreatment technique that enables preparative isolation, fractionation, and purification of peptides from complex mixtures. In food analysis, its application has been reported prior to preparative RP-HPLC or further fractionation techniques (Aito-Inoue et al., 2006). Taking into account the large commercial supply of sorbents in cartridges and columns (e.g., C_{18}, C_8, C_2, phenyl, cyanopropyl and ion-exchange bonded materials, among others), the versatility of SPE has been improved, providing a wide range for peptide isolation and fractionation. However, as shown in Table 3.2, its use appears not to have been significantly extended in recent years. Three mechanisms are offered for peptide isolation: nonpolar, polar, and ion exchange (González de Llano et al., 2004). For example, Li et al. (2010) fractionated peptides from a pea protein hydrolysate by means of cationic exchange SPE cartridges, eluting the peptides with 2% formic acid, methanol and an increasing solution of ammonium hydroxide in Milli-Q water. On the other hand, Hernández-Ledesma et al. (2005) treated the water-soluble extract of fermented milk with a Sep-Pak C-18 cartridge and acetonitrile/water (70:30 v/v) elution. Unfortunately, SPE presents the use of large amounts of organic solvents as a main disadvantage that may be difficult for subsequent analysis.

3.2.2 Peptide Separation

3.2.2.1 Reversed-Phase Chromatography

The most widely used material for the separation of peptides are columns with silica-based reversed-phase (RP) particles, among which the majority of researchers use C_{18} phases. In RP chromatography the retention time increases with increasing peptide hydrophobicity. Numerous examples can be found in a varied number of food matrices (Table 3.3). RP-HPLC has been used to characterize small peptides and amino acids that directly contribute to the flavor of long-term-cured products or contribute indirectly as precursors of other flavor compounds (Setandreu et al., 2003; Mora et al., 2011). Likewise, by using RP-HPLC on a C_{18} column, Sforza et al. (2006) reported the generation of peptides as a consequence of endogenous enzyme action under the conditions of pH, salt content, and moisture in the muscle during meat aging. Paul and Somkuti (2009) studied the degradation of milk-based bioactive peptides by yogurt fermentation bacteria by using also RP-HPLC. The choice of chromatographic column plays a major role in the specificity and sensitivity for the analysis. A C_{18} column packed with superficially porous fused core particles has been used for the separation of six target peptides from gluten. By placing a solid core at the center of the particle, the potential diffusion path length for the peptides through the 0.5 μm porous layer was shortened considerably, compared to that in a totally porous particle (Sealey-Voyksner et al., 2010).

3.2.2.2 Ionic Exchange Chromatography

IEX columns have been used extensively for the separation of peptides and proteins. IEX is mostly used at acidic pH, where all the peptides carry a net positive charge, the separation being based on the effective charge of the peptides. Cation exchange was applied for isolation of meat products peptides, paying attention to prevent the interference from other nitrogen compounds. Meat quality was found to influence

TABLE 3.3

Applications of HPLC in Food Peptide Separation

Food Matrix	Analyte	Separation Mode	Column	Reference
Ham	Sensory peptides	RP	C_{18} Jupiter (4.6 × 250 mm)	Sforza et al. (2006)
Pork meat	ACE inhibitory peptides	RP	Symmetry C_{18} (4.6 × 250 mm)	Escudero et al. (2010a)
Yogur	Bioactive peptides	RP	Vydac C_{18} peptide (4.6 × 250 mm)	Paul and Somkuti (2009)
Consumer products including gluten-free products: flour, bread, pasta, wine, sauces …	Gluten peptides	RP	C_{18} porous core fused particles Ascentis Express (2.1 × 150 mm)	Sealey-Voyksner et al. (2010)
Meat products	Released peptides	IEX	Spherisorb S10SCX (10 × 250 mm)	Moya et al. (2001)
Salmon	Antioxidant peptides	IEX	Macro-Prep High Q (16 × 150 mm)	Wang et al. (2008)
Manchego cheese	ACE inhibitory peptides	IEX	Hi-Load™ Sepharose 26/10	Gómez-Ruiz et al. (2002)
Cheddar cheese with soy protein isolate	Sensory peptides	SEC	TSK gel 2000SWXL (7.8 × 30 mm)	Atia et al. (2004)
Soybean flour	Released peptides	SEC	Superdex™ Peptide HR 10/30	Lee et al. (2001)
Whey protein isolate	Sensory peptides	SEC	TSK G2000SW (7.5 × 600 mm)	Leksrisompong et al. (2010)
Maillard reaction system models	Amadori compounds	HILIC	Atlantis HILIC silica column (2.1 × 50 mm)	Hao et al. (2007)
Goat sweet whey	κ-casein glycomacropeptide	HIC	Phenyl-agarose (15 × 52 mm)	Silva-Hernández et al. (2002)
Bovine serum albumin tryptic digest	Amadori peptides	AC	*m*-Aminophenylboronic acid-agarose (7.5 × 85 mm)	Frolov and Hoffman, (2008)

Note: ACE: angiotensin converting enzyme, RP: reversed-phase chromatography, IEX: ionic exchange chromatography, SEC: size-exclusion chromatography, HILIC: hydrophilic interaction chromatography, HIC: hydrophobic interaction chromatography, AC: affinity chromatography.

the system involved in the production of the final proteolytic products, such as peptides and amino acids (Moya et al., 2001). Gómez-Ruiz et al. (2002) used a cationic exchange column connected to a fast protein liquid chromatography (FPLC) system as a first step to separate and identify ACE inhibitory peptides from Manchego cheese. This mode of separation is very often used in multidimensional separations, as will be shown later.

3.2.2.3 Size-Exclusion Chromatography

The length and structure of the polypeptide chain are used to separate peptides by size-exclusion chromatography (SEC). Soy is a food matrix where this chromatographic mode has been widely used. Lee et al. (2001) used a Superdex column to determine the peptide mass distribution in the soybean hydrolytic process. In a study aimed to evaluate the impact of the addition of soy protein isolate on the proteolysis and organoleptic properties of Cheddar-type cheese during ripening, the use of SEC-HPLC permitted to compare the molecular weight range of the experimental and control cheeses (Atia et al., 2004). In a recent study with different whey protein isolates, correlations between bitter taste intensity, degree of hydrolysis, and concentration of different molecular weight peptides was possible with the fractionation of the peptide fraction with SEC (Leksrisompong et al., 2010).

3.2.2.4 Hydrophilic Interaction Chromatography

Hydrophilic interaction chromatography (HILIC) separates compounds using a polar column and a hydrophobic (mostly organic) mobile phase, where the retention increases with hydrophilicity of solutes, which is the inverse of RP chromatography. The mechanism of separation of HILIC is based on the formation of a water-rich layer on the surface with the polar stationary phase, creating a liquid–liquid extraction system. However, in addition to partitioning of solutes, the mechanism includes hydrogen donor chromatography of neutral polar species, as well as weak electrostatic mechanisms under the high organic solvent conditions used for retention (Issaq et al., 2009). In the peptide field it is not widely used because of the poor solubility of peptides in the high organic content solvents. However, some interesting examples have been reported in the last decade. The importance of temperature in HILIC separation has been highlighted in a study aimed to understand the separation mechanisms for the very polar Maillard reaction components such as amino acids, peptides, and their corresponding Amadori compounds, where a HILIC column was compared to normal-phase silica columns (Hao et al., 2007).

3.2.2.5 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) has proved to be a powerful and very promising technique for purification and separation of complex mixtures. It lies on hydrophobic interactions between immobilized hydrophobic ligands and nonpolar regions on the surface of proteins. Hence, most of the recent applications concern the protein field. In the case of peptides, this type of chromatography has exploited hydrophobicity of bitter peptides, which can be completely removed on hydrophobic stationary phases like hexylepoxy Sepharose of phenolic formaldehyde resins (Careri and Mangia, 2003). HIC has been also applied to the isolation and analysis of κ-casein glycomacropeptide from goat sweet whey (Silva-Hernández et al., 2002).

3.2.2.6 Affinity Chromatography

Affinity chromatography (AC) is a selective purification, separation, and enrichment technique in which the peptides of interest must have a specific property that can be exploited during the affinity procedure. There have been interesting developments in proteomic studies for the enrichment of targeted peptides by choosing an appropriate ligand to be attached to the column. These strategies will definitely enter the food analysis field in the coming years. Enrichment of phosphorylated peptides from peptide mixtures using immobilized metal affinity chromatography (IMAC) is widely used. With this approach the negatively charged phosphorylated peptides are purified by their affinity to metal ions like Fe^{3+} or Ga^{3+} for the selective enrichment of phosphorylated peptides prior to electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis. A promising strategy was introduced by Pinkse et al. (2004) where titanium dioxide (TiO_2) was used as an alternative ligand. Further, Larsen et al. (2005) used dihydroxybenzoic acid (DHB) to enhance the selective enrichment of phosphorylated peptides by TiO_2 adsorption. This novel methodology resulted in a remarkable increase in the selectivity of purification of phosphorylated peptides from complex mixtures of nonphosphorylated and phosphorylated peptides. The authors attribute the enhancement of phosphorylated peptide binding selectivity to an effective competition between DHB and nonphosphorylated peptides for binding sites on TiO_2.

Cysteine (Cys) contains a free thiol (-SH) with unique chemical reactivity that may be exploited for chemical tagging/capture with affinity chromatography allowing specific isolation and subsequent identification of peptide sequences by MS. An affinity matrix suitable for enriching Cys-containing proteins and peptides exploits the reversible interaction of organomercury compounds cross-linked to agarose with thiols. Tryptic peptides from yeast lysates containing Cys were captured and eluted from Hg-beads after incubation with trypsin (Raftery, 2008).

Frequently, it is not possible to directly analyze the glycation sites in a complex peptide mixture. A universal enrichment procedure for glycated peptides using boronic acid AC in the first dimension followed by RP-HPLC was used for the enrichment of glycated peptides (Frolov and Hoffman, 2008).

For bovine serum albumin, a total of 31 Amadori peptides were identified in a tryptic digest corresponding to 26 different glycation sites.

3.2.2.7 Multidimensional Separation

Sometimes, separation of a complex peptide mixture is not possible using a single chromatographic mode. There have been many approaches for the separation of complex peptide mixtures using two or more orthogonal HPLC separation procedures. The most widely used approach employs IEX in the first dimension and RP in the second dimension. The last dimension is usually RP because of its high-resolving power and compatibility of the solvents with MS analysis. Using this strategy, Kim et al. (2010) isolated numerous iron-binding peptides by using anion-exchange chromatography and RP in samples of colostral whey submitted to the action of papain, pepsin, trypsin, and alcalase.

Multidimensional separation of complex mixtures is not limited to IEX in the first dimension and C_{18} RP silica-based columns in the second dimension. For example, Kim et al. (2003) isolated 21 peptides purified from the bitter fraction of tryptic hydrolysates of soybean 11S glycinin by using SEC and a series of three C_{18} HPLC columns. By this approach, the authors were able to determine the amino acid sequence of bitter peptides and identify the position of these peptides in the glycinin molecular structure. Another multimode approach used successively SEC, RP, and IEX to find out the principal antiplatelet peptides of a soy protein hydrolysate (Lee and Kim, 2005). Schlichtherle-Carny et al. (2003) used HILIC for the first time in complex food samples containing peptides such as wheat gluten and Parmesan cheese. After a SEC fractionation and further sub-fractionation by RP-HPLC, the compounds of the void volume peak not retained by RP were separated and characterized by HILIC-ESI-MS/MS. In a more recent report, consecutive chromatographic methods including IEX, SEC, and a two-step RP-HPLC were used to purify an antioxidant peptide from loach protein (You et al., 2010). A different combination was used by Cheng et al. (2010a,b) to identify the main peptides present in antioxidative potato protein hydrolysate. They separated the peptides into different molecular weight fractions using SEC followed by purification with RP-HPLC. The individual peptides were subsequently purified using ultraperformance liquid chromatography (UPLC), a new technique that permits the use of smaller particles and shorter columns at higher flow rates. This combination reduces considerably the time of analysis.

3.2.2.8 Miniaturized Techniques

The role of miniaturized techniques such as capillary electromigration methods (which also include capillary electromigration chromatography, CEC), microchip and nano-LC/capillary LC (CLC) in food analysis is still probably in an early stage. Nevertheless, these techniques offer several advantages over classical methods, such as reduced consumption of mobile and stationary phases, short analysis time, easy coupling with MS and low environmental pollution (see Table 3.4). When liquid chromatography is performed in capillary columns of internal diameter (id) in the range between 10 and 100 μm, the technique is named nano-LC, while when making use of higher id columns (100–500 μm) the method is called CLC. In both modalities, liquid chromatography columns contain either silica-modified particles of 3–5 μm, monolithic supports or wall coated with appropriate materials (Asensio-Ramos et al., 2009).

Two species of the genus Coffea, *Coffea arabica* (Colombia) and *Coffea canephora* (Indiano Robusta) were analyzed by two-dimensional (2-D) maps in order to obtain fingerprints of the expressed polypeptide chains and to determine which ones would characterize the two species. Separation was performed in the nano-LC mode using a homemade RP capillary column (Gil-Agusti et al., 2005). Another application includes the combination of RP-CLC with quadrupole time-of-flight tandem mass spectrometry (nano-ESI Q-TOF MS/MS) developed with the aim of identifying a set of peptides that can function as markers for peanut allergens (Chassaigne et al., 2007). An efficient approach to eliminate gluten toxicity is to submit wheat flour to lactobacilli and fungal proteases during long-time fermentation. Strong-cation-exchange-LC/CLC-ESI-Q-TOF analyses were used to determine the gluten concentration in samples where the residual concentration of gluten was 12 ppm (Rizello et al., 2007). **Q3**

Monolithic columns offer advantages of eliminating the technical problems of frits to retain the packed bed, while providing a stable chromatographic bed, adjustable pore diameter, and low column back

TABLE 3.4

Food Peptide Separation by Miniaturized Methodologies

Food Matrix	Analyte	Separation Mode	Column	Reference
Coffea	Polypeptides	NanoLC (RP)	Zorbax 300 SB, C_{18} (75 μm × 100 mm)	Gil-Agustí et al. (2005)
Peanut	Allergenic peptides	CLC (RP)	Grace Vydac C_{18} (50 μm × 150 mm)	Chassaigne et al. (2007)
Wheat flour	Gluten peptides	CLC (IEX)	SCX BIO BASIC	Rizello et al. (2007)
Casein	Phosphopeptides	NanoLC (RP)	C_{18} Pep Map 100 (75 μm × 150 mm)	Zhu and FitzGerald (2010)
Ham	Muscle peptides	NanoLC (RP)	Dionex C_{18} PepMap (75 μm × 150 mm)	Mora et al. (2011)
Egg and milk protein digests	Phosphopeptides	NanoLC (AC)	Monolithic Column with Hydroxyapatite Nanoparticles (100 μm id)	Krenkova et al. (2010)

Note: CLC: capillary liquid chromatography, RP: reversed phase chromatography, IEX: ionic exchange chromatography.

pressure under high eluent flows. Making the packing material in a monolithic format suggests a new trend for capillary columns. In this context, Huang et al. (2002) described a simple process for alkylating flow-contacting surfaces of a macroporous poly(styrene-divinylbenzene) (PS-DVB) monolith. This synthetic polymer is very effective for the rapid analysis of proteins but, in terms of peptide separation, it usually provides relatively poor chromatographic resolution. The process introduced by these authors provided better results with respect to those achieved using an unmodified monolithic column. Kimura et al. (2004) employed IEX in the first dimension and compared conventional and capillary octadeylsilylated monolithic silica columns to resolve the peptides of a bovine serum albumin digest. The use of a capillary column in the second dimension led to less solvent consumption and better MS detectability compared to a larger-sized column. A different strategy consists on the use of monolithic capillary columns with embedded commercial hydroxyapatite nanoparticles. Selective enrichment of phosphopeptides from complex peptide mixtures of ovalbumin, α-casein, and β-casein digests have been achieved with these columns (Krenkova et al., 2010).

3.2.3 Peptide Detection

The correct choice of a separation technique for peptide analysis has to be followed by the adequate detection system. Among the different methods developed for the analysis of peptides in food matrices stand out mainly three: UV absorbance detection (the most commonly used), fluorescence detection and MS detection. Over the last 10–15 years, the initial development of MS techniques has been followed by many advances in this area, and it has converted the others detection modes in secondary actors.

3.2.3.1 Absorbance Detection

Far UV detection has been traditionally the most widely used detection method for peptide analysis. Despite the proliferation of MS techniques, UV detection is still applied for peptide analysis, generally as first detection mode before sample ends in the mass spectrometer. In many cases, UV detection is used during the intermediate chromatographic steps that are needed to obtain more or less pure fractions before carrying out biological experiments and MS detection (Quirós et al., 2005, 2007). A recent publication also shows the combination of SEC with UV detection to characterize the molecular mass distribution of peptides in commercial protein hydrolysates and in protein hydrolysate-based liquid nutritional products (Johns et al., 2011).

The maximum absorbance for the peptide bond is around 185 nm, but many food components as well as solvents used for the analysis absorb at that wavelength. Therefore, a wavelength between 210 and

220 nm is usually selected, although most peptides show higher sensitivity at 210 nm. Besides the peptide bond, some amino acids make also an important contribution to the absorbance at these wavelengths. It is the case of tryptophan (Trp) with a molar extinction coefficient at 214 nm 30 times higher than that of the peptide bond (Kuipers and Gruppen, 2007).

Peptides with aromatic residues, Trp, tyrosine (Tyr) and phenylalanine (Phe), can also be detected at 278–280 nm, although Phe has higher absorbance at 254 nm than at 280 nm. The inclusion of UV detectors based on the simultaneous detection of more than one wavelength served to confirm the identity of peptides that contain aromatic residues. Detection of aromatic amino acids-containing peptides is very useful for detection of bioactive peptides since the occurrence of these amino acids in their sequence is related with their biological activity. This feature has been exploited to isolate bioactive peptides, for example, ACE inhibitory peptides in complex matrices such as Manchego cheese (Gómez-Ruiz et al., 2002). Furthermore, the use of photodiode detectors enables the use of derivative spectrophotometry based on the different absorption minima of the different second- and fourth-order derivatives of each aromatic amino acid. This approach has been used for the identification of β-casein and the components of its plasmin hydrolysate (Darewicz et al., 2005) as well as the evaluation of the exposition extent of aromatic amino acids during protein hydrolysis and the determination of the extent of encapsulation of casein hydrolysates (Barbosa et al., 2004).

To overcome the disadvantages of working at wavelengths below 220 nm, peptide derivatives that absorb at higher wavelengths can be generated. There are many derivatizating agents that can be used, among them ninhydrin (derivatized peptides absorbing at 570 nm), phenyl isothiocyanate (PITC) and dansyl chloride (both derivatives absorbing at 254 nm), and *o*-phthalaldehyde (OPA) whose derivatives absorb at 340 nm. Some of these reagents like dansyl chloride and OPA also allow peptide detection by means of fluorescence detectors. For instance, dansyl chloride is detected at excitation wavelength of 320–360 nm and at 430–470 nm of emission wavelength. Concretely, dansyl chloride has become the preferred derivatization strategy to determine biogenic amines in food by means of fluorescent detection since this compound reacts with both primary and secondary amino groups providing very stable derivatives (Loukou and Zotou, 2003).

3.2.3.2 Fluorescence Detection

Fluorescence spectroscopy is generally 1–3 orders of magnitude more sensitive than corresponding absorption spectroscopy methods. In some cases, it is possible to use the natural fluorescence of certain amino acids. The native fluorescence of Trp and Tyr in the 210–290 nm range (with the highest sensitivity at 220 nm) can be exploited for peptide determination with high picomolar to low nanomolar limits of detection. The highest emission is obtained at 320–360 nm. However, many peptides do not contain these amino acids within their sequences and they cannot by detected by this method. To overcome this problem different fluorescent molecules are used to derivatize the peptides before the analysis. Some of the compounds used for derivatization include fluorescamine or OPA but also naphthalene-2,3-dicarboxaldehyde (NDA), fluorescein isothiocyanate (FITC) and 3-(4-carboxybenzoyl)2-quinolinecarboxaldehyde (CBQCA), which react not only with primary but also with secondary amines (Table 3.5).

OPA reacts with primary amines at alkaline pH (9–11) in the presence of a thiol group. The isoindole formed has to be stabilized in acid medium and can be detected by flourescence (excitation at 340 nm, emission at 455 nm). The presence of lysine (Lys) provides much greater fluorescence intensity (up to 50 times more) than peptides without Lys. This reagent has been widely used in the past in postcolumn and precolumn derivatization in food analysis (Acedo et al., 1994; Herráiz, 1994). However, the poor **Q4** stability of its derivatives limits its application and new but similar reagents have been synthesized. An example is the reagent NDA, whose derivatives have excitation/emission maxima of ~403/500 nm. Derivatives generated with NDA using precolumn fluorescent derivatization show higher sensitivity and stability than those generated with OPA (Rammouz et al., 2007). Due to the high sensitivities reached with the NDA derivatives, this method has been mainly used for the detection of different compounds in biological samples such as opioid peptides and food peptides (Yang et al., 2002; Rammouz et al., 2007).

TABLE 3.5

Most Typical Methods Used for Peptide Detection with HPLC Analysis

Method of Detection	Characteristics	References
Absorbance		
UV (210–220 nm)	Most common method for peptide detection in HPLC. Contribution of peptide bond and amino acids.	Kuipers and Gruppen (2007)
UV (254 and 280 nm)	Detection of peptides with aromatic amino acids (Tyr, Trp, Phe). Useful for detection of bioactive peptides.	Gómez-Ruiz et al. (2002)
Derivatization with ninhydrin (570 nm)	Used for post-column detection of peptides and amino acids.	Friedman (2004)
Derivatization with phenyl isothiocyanate (254 nm)	It reacts with primary and secondary amino groups. Used for peptide sequencing by Edman degradation.	Smith (2001)
Derivatization with dansyl choride (254 nm)	Derivatives also detected with fluorescence detectors. It reacts with primary and secondary amino groups. Very stable derivatives.	Loukou and Zotou (2003)
Fluorescence		
Native fluorescence of Trp and Tyr	Detection of peptides with Trp and Tyr. Exc. 210–290 nm; Em. 320–360 nm.	Li and Seeger (2010b)
Derivatization with OPA	Widely used in the past for food peptide analysis. Exc. 340 nm; Em. 455nm. Derivatives also detected at absorbance of 340 nm.	Herráiz (1994); Acedo et al. (1994)
Derivatization with NDA	Higher sensibility and stability than OPA. Exc.403 nm; Em. 500 nm.	Rammoouz et al. (2007)
Derivatization with FITC	Often used in cellular biology to label and track cells. Exc.447 nm; Em. 514 nm.	Huang et al. (2005)
Mass Spectrometry		
Ionisation Modes		
	Mass spectrometers offer structural information.	Kebarle and Verkerk (2009); Wysocki et al. (2005)
FAB	ESI produces multiply charged ions and allow the analysis of proteins. Main ionization technique used in HPLC-MS/MS analysis.	
ESI	FAB is limited in the low-mass range by matrix-associated chemical noise.	
APCI	APCI is typically used to analyze small peptides with molecular weight up to 2000 Da.	
Analyzers		
Quadrupole (Q)	Q-IT: for qualitative applications. It can conduct MS^n experiments.	Hopfgartner et al. (2004); Lacorte and Fernández-Alba (2006)
Time of flight (TOF)	Q-q-Q: best suited for quantitative analysis.	
Ion trap (IT)	Q-TOF: highest mass accuracy.	
Fourier-transform ion cyclotron resonance (FTICR)		

Note: UV: ultraviolet, OPA: *o*-phthalaldehyde, NDA: naphthalene-2,3-dicarboxaldehyde, FITC: fluorescein isothiocyanate, FAB: fast atom bombardment, ESI: electrospray ionisation, APCI: atmospheric-pressure chemical ionization.

3.2.3.3 Mass Spectrometry Detection

Today MS techniques play a key and determinant role in the analysis of peptides and proteins as shown by different reviews (Careri and Mangia, 2003; Wysocki et al., 2005; Mamone et al., 2009). These techniques are among the most sensitive, and they provide specificity, speed, and reliability of the analytical response when working at high sample throughput. In addition, MS not only carries out peptide detection but also provides information of its structure.

Mass spectrometers usually comprise several major components with the sample inlet, the ion source, and the mass analyzer defining the type of instrument and the capability of the system. Considering HPLC as our inlet system, in this chapter we will focus on the different ionization techniques and mass analyzers used for peptide analysis.

3.2.3.3.1 Ionization Techniques

The use of MS techniques in food peptide analysis experienced an important boost since the introduction of soft ionization techniques such as fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI). FAB ionization is one of the pioneering desorption techniques that revolutionized the analysis of food peptides and small proteins by MS. This ionization mode requires the use of a direct insertion probe for sample introduction and uses a high-energy beam of Xe, Ar, or Cs atoms, among others, to sputter the sample and matrix from the probe surface. FAB ionization has been widely applied for the analysis of bioactive peptides such as phosphopeptides, antihypertensive, or opioid peptides (for a comprehensive review, see Contreras et al., 2008). These studies were mainly focused on dairy products, although peptides from different foodstuff have also been analyzed by FAB-MS, for example, antihypertensive peptides from wakame (Suetsuna et al., 2004), antioxidant peptides in prawns (Suetsuna, 2000), or pyroglutamyl peptides in wheat gluten (Higaki-Sato et al., 2003). However, the limitations of FAB ionization, mainly related the range of molecular masses accessible, provoked the development of other soft ionization techniques and the authentic revolution in MS analysis of biomolecules. As a result, ESI and MALDI emerged as two soft ionization techniques that provided ionization without fragmentation, accurate mass determination, picomole to femtomole sensitivity and broad applicability.

ESI technique allows the transfer of the analyte, generally ionized in the condensed phase, into the gas phase as isolated entities. In a first step, the solution that contains the peptides is sprayed through a small-diameter needle held at high potential. As the solution emerges a mist of highly positive charged droplets is generated and they are moved from the needle to the negatively charged instrument. During this transition, evaporation reduces the size of the droplets by "Coulomb explosion" (droplet subdivision resulting from the high charge density) before the fully desolvated ions move into the high vacuum of the mass spectrometer (Kebarle and Verkerk, 2009). ESI usually produces a population of multiply charged ions; in the case of peptides and working in positive mode, the number of charged species observed mainly depends on the number of arginine (Arg) or Lys residues protonated at low pH (Figure 3.1a). In the case of MALDI technique, samples are co-crystallized onto a sample plate with different organic matrixes (α-cyano-4-hydroxycinnamic acid, sinapic acid, etc.). Pulses of UV laser light are used to vaporize small amounts of the matrix and the incorporated peptide ions are carried into the gas phase in the process. Matrix excess is required because it is the matrix crystals that absorb the laser ultraviolet light. Although multiply charged ions can also be generated, only single charged ions are normally generated in MALDI experiments. ESI technique has become the predominant ionization method for HPLC-MS analysis due to the fact that MALDI cannot be adapted to chromatographic interfacing because the samples are solid. Other ionization techniques as atmospheric pressure chemical ionization (APCI) have a minor use for the analysis of food peptides, although this technique was used, for example, for the analysis of free amino acids and peptides in dry-cured Iberian hams (Martín et al., 2001).

3.2.3.3.2 Mass Analyzers

The combination of ESI and MALDI techniques with different mass analyzers has stimulated the development of a great variety of MS instruments. There are mainly four different mass analyzers in the market with application in peptide/protein analysis: quadrupole (Q), time of flight (TOF), ion trap (quadrupole ion trap 3D, QIT; linear ion trap, LIT or LTQ), and Fourier-transform ion cyclotron resonance (FTICR). The MS instrument can provide information on the mass of a particular peptide based on its *m/z* (mass–to-charge ratio), but it can also carry out MS/MS. This term refers to the fragmentation of a parent ion, usually by collision-induced dissociation (CID) with neutral gas, to produce daughter ions that are analyzed to obtain the amino acid sequence of a specific peptide (Figure 3.1b). Mass spectrometers can be also classified according to how MS/MS is accomplished: tandem-in-space or tandem-in-time. In tandem-in-space instruments the selection of parent ion, its fragmentation and the analysis of daughter ions occur sequentially in separate regions of the instrument. These instruments possess more than one mass analyzer, and are for instance, tandem quadrupole (e.g., triple quadrupole (Q-q-Q), Q-TOF, reflectron-time-of-flight, tandem sector, and sector-quadrupole. On the other hand, in tandem-in-time instruments all these processes occur sequentially in the same physical space, with only one mass analyzer. QIT or FTICR instruments are examples of this type of systems.

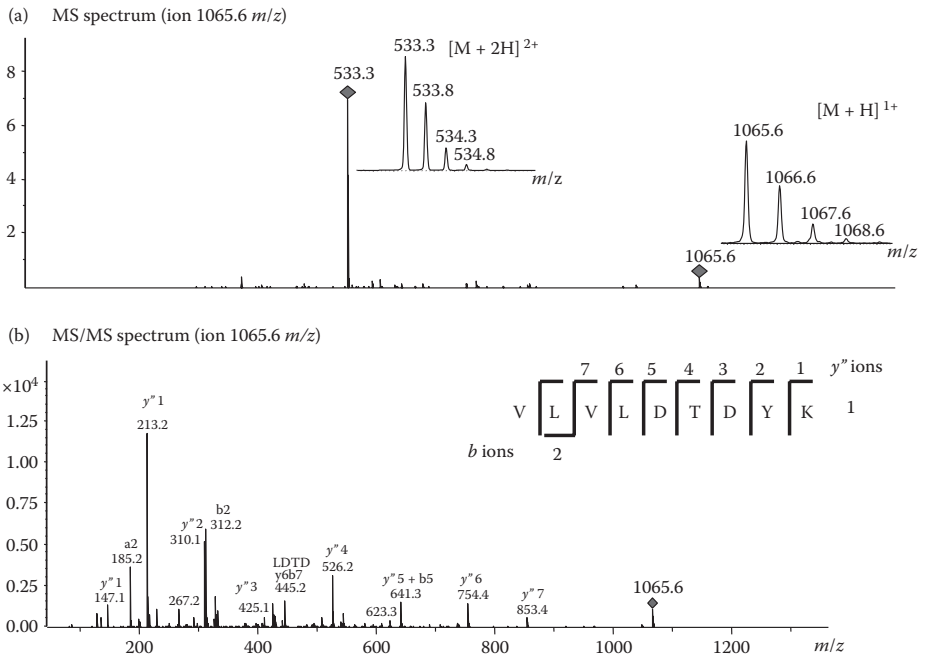

FIGURE 3.1 (a) ESI-q-TOF spectrum obtained from a peptide with molecular weight of 1064.6. Single charged and doubled charged ions are detected in the spectrum. (b) MS/MS spectrum of the single charged ion 1065.6 Following sequence interpretation and database searching peptide was identified as β-Lg f(92–100). (D. Martínez-Maqueda et al., unpublished results.)

Q10

3.2.3.3.3 Ionization Source-Mass Analyzer

Among the different configurations *ionization source-mass analyzer* the most common are: ESI-Q-q-Q, ESI-QIT, ESI-QTOF, MALDI-TOF, and MALDI-TOF/TOF. Focusing on ESI technique, this ionization mode is used with the most common mass analyzers. ESI-Q-q-Q, ESI-QIT, and ESI-QTOF have been applied to characterize the proteome and the peptidome of a big variety of foodstuff, providing information on the biological activity of food peptides, allergenicity, and so on (Minkiewicz et al., 2008; Mamone et al., 2009). The ability of ESI to produce multiply charged ions enables the analysis of large peptides and proteins even when ESI is coupled to quadrupoles which are usually limited in mass range (Kochhar et al, 2001; Monaci and van Hengel, 2007).

The type of research carried out in the laboratory together with the economic possibilities will determine the choice of one configuration or another. Each configuration offers different capabilities: QIT has mostly been used for qualitative applications and can conduct MS^n experiments, Q-q-Q is the best suited for quantitative analysis, and QTOF analyzers provide the highest mass accuracy (Hopfgartner et al., 2004; Lacorte and Fernandez-Alba, 2006).

3.3 Other Detection Methods

Some other detection techniques have been proposed in HPLC systems for the detection of peptides, among them electrochemical detection, chemiluminiscence detection or evaporative light-scattering detection. However, their application to food peptide analysis is irrelevant and will not be treated in this chapter.

3.3.1 Applications: Food Peptidomics

Food peptidome has been defined as the whole peptide pool present in food products or raw material, as well as peptides obtained during technological processes and storage (Minkiewicz et al., 2008). As reported by these authors, food peptidomics covers research concerning the origin of peptidome, its dynamic changes during processing and/or storage, influence of its presence, composition and changes on the properties of food products or raw material. Several areas of interest have been established for food peptidomics. They include biological activity, functional and sensory properties, allergenicity, and information on the product or resource authenticity and origin as well as its history and relationships.

3.3.1.1 Biologically Active Peptides

Food protein digests can have functional, nutritional, and biological applications. Functional applications include improved whipping, gelling, solubility, or acid stability of formulated products. Nutritional applications include increased digestibility *in vivo* and reduced allergenicity. During the last decades, several studies have shown that food protein hydrolysates or peptides generated by hydrolysis possess also biological properties. These peptides, inactive within the sequence of parent protein, are activated once released during gastrointestinal digestion or during food processing (Meisel, 2005). Once they are released in the body, bioactive peptides may act as regulatory compounds with hormone-like activity, exhibiting a wide range of biological functions, including antihypertensive, antioxidant, opioid, antimicrobial and immunostimulating activities (Yalcin, 2006; Hernández-Ledesma et al., 2008; Madureira et al., 2010). Due to their physiological and physiochemical versatility, food-derived bioactive peptides are regarded as highly prominent components for health-promoting foods or pharmaceutical applications. Intensive efforts are now focused on identification and quantification of these bioactive peptides in the developed food ingredients, in the final functional food, and in the organism to determine absorption, degradation, and metabolism (Contreras et al., 2008). As recently reviewed by Mamone et al. (2009) analytical research on food bioactive peptides requires the development and validation of methods for (1) tracing the pathway of formation of bioactive peptides from the parent proteins; (2) evaluating the biological properties; and (3) designing of synthetic structural analogues to improve the properties of natural peptides. Figure 3.2 shows the workflow generally used to identify and characterize bioactive food peptides. The potential bioactive peptides are present in the processed food or may be generated by hydrolysis of total or individual food proteins. A first separation phase is required to isolate and purify these peptides (phase 1, Figure 3.2) from the complex mixture. High-resolution techniques or selective methods for enrichment of some food components are currently being used to complete this process. IEX is a routinely used technique to isolate bioactive peptides from different food sources (Bizani et al., 2005; Rui, 2009; Wu et al., 2009; Kannan et al., 2010). In the last few years, combination of this technique with other chromatographic methodologies, such as RP-HPLC, SEC, or FPLC has become an efficient tool to separate food peptides with calcium-binding (Kim and Lim, 2004), antioxidant (Wang et al., 2008), and ACE inhibitory properties (Guan et al., 2009). There is a growing demand for effective sample preparation strategies that allows enriching food phosphopeptides where mineral bioavailability enhancing and antioxidant properties are well known (Kansci et al., 2004; Gravaghi et al., 2007). Currently, metal oxide affinity chromatography is becoming one of the most widely used strategies in "phosphopeptidomics" (Leitner, 2010).

Once separated, peptides are subjected to an identification and quantification process (phase 2, Figure 3.2), requiring peptidomic approaches combining high-resolution separation techniques with MS, and database cataloguing. Due to its specificity and sensitivity, especially in combination with HPLC, MS has become the indispensable analytical tool for identification, quantification, and bioavailability of health-promoting peptides (Careri and Mangia, 2003; Contreras et al., 2008). In particular, the improved resolution and sensitivity of the equipment, MALDI, nano-ESI, and the introduction of hybrid mass spectrometers into one instrument will provide even better sensitivity and a simplification in sample preparation due to the high selectivity. Identification of phosphopeptides is a challenge because of their poor ionization efficiency and low abundance (Garcia et al., 2005). Among different mass analyzers suitable for peptide **Q3** identification, qTOF instruments have shown to display good resolution, mass accuracy, and sensitivity (Forner et al., 2007). Recently, Zhu and Fitzgerald (2010) have carried out a nano-HPLC-ESI-QTOF MS/MS

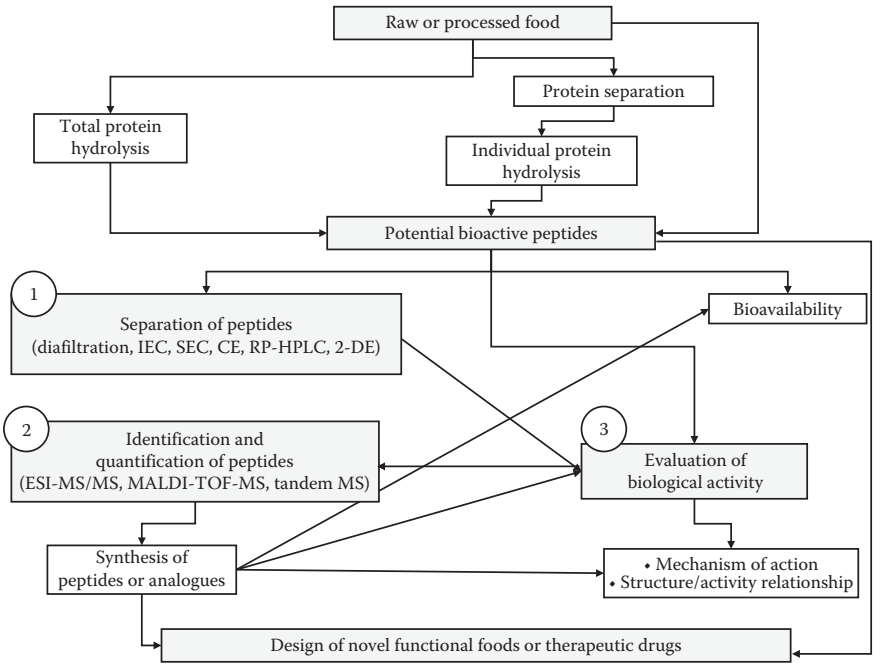

FIGURE 3.2 Workflow illustrating integrated strategies for identification and characterization of bioactive peptides derived from foods.

methodology for direct determination of caseinophosphopeptides in enriched casein hydrolysates. Similarly, different chromatography/MS strategies are being developed and optimized to automatically identify and quantify bioactive peptides from protein hydrolysates without any previous sample treatment (Butikofer et al., 2007; Ferreira et al., 2007; Bougatef et al., 2010; Contreras et al., 2010).

Identified peptides and analogues are synthesized to carry out further studies focused on evaluating their potential biological activity (phase 3, Figure 3.2), their structure–activity relationship and mechanism of action, as well as their bioavailability. Convenient, reliable, and sensitive methods are needed for determining the biological activities. Chromatographic techniques have shown to be a promising strategy to evaluate ACE inhibitory activity of peptides derived from different food sources, and therefore, different HPLC methods are currently being used (Liu et al., 2010a; Lahogue et al., 2010). Moreover, the rapid and sensitive method using UPLC-MS recently developed and applied to assay the ACE inhibitory activity of natural phenolic compounds might be a good alternative for measuring this activity in food-derived peptides (Geng et al., 2010).

Information obtained from previous studies is essential for the design and production of functional foods ingredients or novel therapeutic drugs. As an example, Contreras et al. (2011) optimized, by using a response surface methodology, the production of a potent antioxidant hydrolyzate where potential bioactive peptides were identified by RP-HPLC/MS/MS.

3.3.1.2 Sensory and Functional Properties of Peptides

Characterization of food components which give a food its particular functional and sensory properties, such as taste, odor, and flavor, has become one of the main areas of research in food technology. This area, defined as "sensomics," has made a great effort to map the complete food sensometabolome, as well as to identify and quantify the most intense taste-active metabolites in fresh and processed foods

(Toelstede and Hofmann, 2008). Over the past years, multiple studies have been conducted to characterize the key taste peptides of different foods. However, the results published in the literature are controversial, particularly in the case of fermented products (meat and dairy foods, wine and beer) where sensory and functional peptides are generated by a complex series of metabolic and technological processes. Low quantity of peptides contained in those foods, as well as their presence in an extremely complex mixture, together with proteins, amino acids, and a multitude of peptide-unrelated substances have made needed the development of new strategies for isolation, identification and/or characterization of these peptides. The "sensometabolomic" approach consists of a combination of chromatographic techniques, with MS analysis and analytical sensorial tools. Desportes et al. (2000) developed a procedure for the isolation of wine small peptides using ultrafiltration, nanofiltration, low-pressure chromatography on Sephadex LH20, and HPLC on a porous graphitic carbon column. Free-solution capillary electrophoresis was used by these authors as a valuable tool to determine the peptide purity subsequent to HPLC fraction-isolation and prior to peptide sequencing. A taste dilution analysis coupled with RP-HPLC has been proven to be a powerful method to screen the key components, including peptides that contribute to food tastes (Lioe et al., 2006, 2007). Recently, Toelstede and Hofmann (2008, 2009) have developed a sensomics approach using LC-MS-TOF and LC-MS/MS, independent synthesis, and sensory analysis to identify sequence of peptides generated by proteolysis of caseins and responsible for bitter, umami and kokumi taste of Gouda cheese.

3.3.1.3 Allergenicity of Food Peptides and Proteins

Food allergy is an immunoglobulin IgE-mediated adverse reaction to some food proteins. There is no treatment available for this disease, thus an allergic reaction only is preventable avoiding the offending food. As a consequence, an accurate food labeling is required. In 2003, the European Union created a list with the major food allergens that was updated in 2007. Currently, 13 food allergen groups, with a total of 26 protein-based allergens are included in that list. Despite this regulation, total avoidance may be difficult for the consumer prone to allergy because of the possible contamination of food with allergens during the manufacturing process. Development of analytical methods able to detect these so-called "hidden allergens" is needed. Chromatographic techniques coupled to MS are being optimized to detect multiple allergens in the same analysis. Some strategies target the intact protein, but in the last few years, approaches targeting the peptides resulting from tryptic digest of an allergen are becoming more usual. Weber et al. (2006) and Monaci et al. (2010) identified peptide markers for the detection of milk in cookies and caseins in white wines, respectively. Detection by MS of peanut in chocolate has been reported by Shefcheck et al. (2006). Sealey-Voyksner et al. (2010) developed a novel, specific, and sensitive non-immunological LC-MS-based assay to detect and quantify trace levels of wheat gluten in food products by the unambiguous identification and structural characterization of six unique physiologically relevant wheat gluten peptides. A recent work describes for the first time a real multi-screening method for seven allergenic targets contained in a bread matrix (Heick et al., 2011). This method is based on extraction of the allergen from the food matrix, enzymatic cleavage with trypsin, separation of peptides by HPLC and peptide identification with a triple-quadrupole MS in multiple reaction mode to enhance sensitivity.

3.3.1.4 Authenticity, Origin, and History of Food Products

To ensure food safety for human consumption at every stage of production, the European Food Safety Authority (EFSA) has established a comprehensive system of authentication and traceability of food and feed that has been defined as "Protected Designation of Origin" (PDO) (Council Regulation, 2006). Quality labels specifying the chemical composition of each product are required to achieve the proposed quality standards. Moreover, with the aim to document the history of a product along the entire production chain from primary raw materials to the final consumable product, an integrated traceability control system should be able to identify with precision ingredients, processing and production methods applied in food chain (Peres et al., 2007). In most cases, quality labels must also contain information about the geographical origin and production methods. These requirements have prompted an increased interest in developing analytical methods to assess quality, authenticity, and traceability of food products. Addition

of cheap plant proteins to skim milk powders used as feed or as ingredients acting as emulsifiers, texturizers in dairy and meat products is a common but illegal practice. Because of the complexity of the matrices and the low amounts of adulteration used, application of traditional analytical methods (HPLC, electrophoresis) is completely unfeasible. Recently developed proteomic methodologies based on combined chromatographic and MS methods open up new possibilities in the routine control of skim milk powders. Two LC/ESI–MS/MS methods have been applied to detect peptides originated from the major soybean and pea proteins in this product (Luykx et al., 2007; Cordewener et al., 2009). Intensive research has focused on the discovery of appropriate markers and relative methods for detecting adulterating milks/cheeses. Recently, liquid chromatography/MS based methods are being developed to detect "signature peptides," corresponding to the predefined subset of "proteotypic peptides," as matchless analytical surrogates of the parent caseins (Cuollo et al., 2010). Similarly, a peptidomic approach, based on LC/ESI-MS/MS has been developed to detect adulteration of goat and cow's cheeses with sheep milk (Guarino et al., 2010).

Biological, analytical, and informatics tools have been synergistically proposed and utilized for traceability in the wine industry (Pinder and Meredith, 2003). The methodologies applied for wine protein characterization and identification include electrophoretic methods (native-PAGE, SDS–PAGE, IEF and CE, 2D-PAGE), chromatographic methods (FPLC, SEC, RP–HPLC), and MS techniques (Moreno-Arribas et al., 2002). Recent investigations are focused on the application of peptide profiles obtained from whole wine protein tryptic digests as a new method valid to wine authenticity and traceability. As an example, Chambery et al. (2009) applied MALDI-TOF-MS methodology to recognize peptide profiles derived from whole protein tryptic digests and obtain a reliable signature of high-quality Campania white wines.

3.3.2 Future Prospects

HPLC in all its formats is an essential analytical technique for peptides separation in simple and complex mixtures using single and multidimensional approaches. Advances in MS will continue to be the corner stone for peptides in foods as well as in hydrolysates for sequence determination. It is expected that the quantification of bioactive peptides by HPLC-MS will be widely applied. Automatization in sample preparation and its online coupling with the advanced high resolution and detection techniques is another point for future development in food peptides analysis. In the near future, HPLC will continue to grow through the miniaturation of the components, and techniques for peptide separations on microchips will progress, especially for proteomic applications. It is to be expected that CLC, chip and nano-LC will establish themselves as complementary and/or competitive separation techniques to conventional LC in peptide separation.

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Chapter 2
Extraction/Fractionation Techniques for Proteins and Peptides and Protein Digestion

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2.1 Introduction

The use of proteomics approaches is a powerful tool in food science in terms of process optimization and monitoring, quality, traceability, safety, and nutritional assessment (Pedreschi et al. 2010). Proteins, together with peptides, are one of the major groups of food components, and they are found in many different organisms of both vegetal and animal origin. Peptides are also obtained during technological processes such as fermentation and storage of foods. Moreover, many experiments involve enzymatic hydrolysis of proteins from food resources such as milk, meat, fish, eggs, or plants to produce a variety of peptides (Minkiewicz et al. 2008).

The study of the food proteome at any specific time is extremely complex and diverse. The major limitations of proteome analysis are, in general, associated with the heterogeneity of proteins and peptides in terms of physicochemical properties and the vast differences in abundance. A typical proteomics workflow consists of (1) protein extraction, (2) protein or peptide separation and quantification, (3) protein identification, and (4) data analysis and interpretation (Carpentier et al. 2008). Sample preparation has a profound effect on the final outcome of protein and peptide separation and their subsequent analysis. These procedures need to be compatible with posterior analysis by two-dimensional electrophoresis (2DE) and/or liquid chromatography tandem mass spectrometry (LC-MS/MS). Therefore, sample preparation should include the steps needed to isolate and fractionate proteins and

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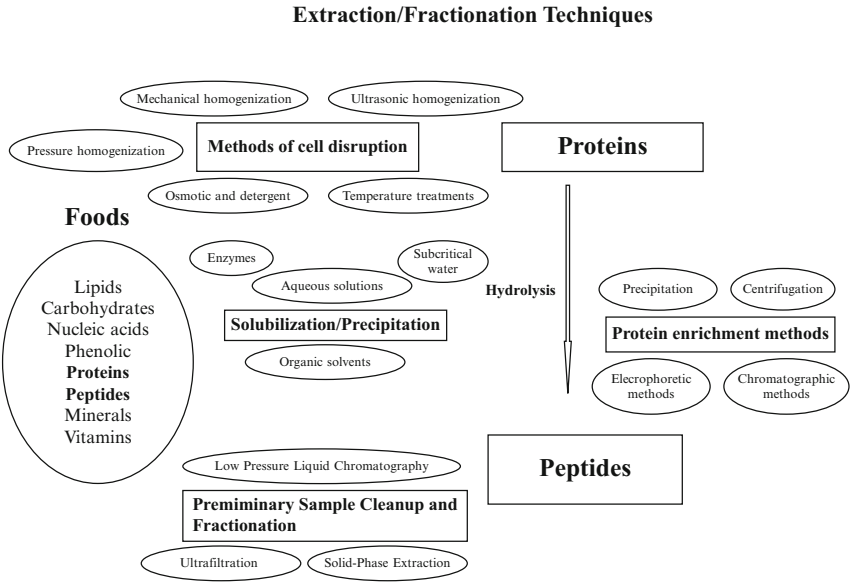

Fig. 2.1 Scheme illustrating integrated extraction and fractionation techniques for proteins and peptides employed on proteomics in foods

peptides ensuring an unbiased reliable map that gives an accurate representation of all proteins and peptides initially present in a particular food.

A wide variety of extraction and fractionation tools for proteins and peptides are available based on their physicochemical and structural characteristics such as solubility, hydrophobicity, molecular weight, isoelectric point (pI), and so on. Figure 2.1 shows an integrated view of extraction and fractionation techniques for proteins and peptides used in food proteomics studies. Generally, different technologies focused on cell disruption, solubilization/precipitation, and enrichment systems are needed to obtain the protein fraction of interest. Removal of interfering compounds (mainly lipids, nucleic acids, phenolic compounds, carbohydrates, proteolytic and oxidative enzymes, and pigments) is crucial. These procedures need to be optimized to minimize proteins´ modifications and proteolysis, as well as to be compatible with subsequent analysis.

This chapter describes the state-of-the-art of extraction and fractionation techniques for food proteins and peptides as a first step prior to proteome studies. The first part is dedicated to classical and novel extraction and fractionation techniques for food proteins, followed by a brief description on protein enzymatic digestion. The second part provides information about several extraction/fractionation techniques mainly used for food peptides.

2.2 Food Protein Extraction and Fractionation

2.2.1 Cell Disruption Methods

The preparation of any biological material as a sample for proteomic analysis requires homogenization. Plants are generally more problematic for protein extraction because tissues are rich in proteases and other interfering compounds (Wang et al. 2008a). Proteins are usually contained in protein bodies inside cell walls so cell disruption is required before they can be totally solubilized and extracted. The general procedure for sample preparation in this case strongly depends on the plant type, its fragment (leaf, fruit, seed, etc.), or even the stage of plant development. Generally, disruption of the cell wall and protein release is crucial for analytical success. Various chemical and physical techniques can be used to destroy the cell wall. These techniques can be grouped into five major categories: mechanical homogenization, ultrasound homogenization, pressure homogenization, temperature treatments, and osmotic and chemical lysis. A summary of these methods with their applications in different food matrices is shown in Table 2.1.

2.2.1.1 Mechanical Homogenization

Mechanical homogenization can be realized by at least two types of devices: so-called rotor–stator homogenizers and open blade mills. Rotor–stator homogenizers are one of the best homogenizing tools applied in laboratories. To homogenize dry samples using mechanical processing, open blade homogenizers, also called blenders, are used (Bodzon-Kulakowska et al. 2007). In the case of plant tissues, where cells are covered with strong cell walls, mechanical homogenization seems to be one of the best methods for disruption (Van Het Hof et al. 2000). Anderson and Guraya (2001) evaluated the use of colloid milling and homogenization to effect bran breakdown and extract rice protein. They demonstrated that the shearing actions of colloid milling and homogenization did not result in any significant denaturation of the proteins. Sometimes, a combination of mechanical homogenization with buffers is used. Examples of this are found in rice (Fukuda et al. 2003) and in olive tree seeds (Alche et al. 2006).

Wet-milling is a physicochemical separation of the components of grain, namely germ, bran, fiber, starch, and protein. Chemicals and enzymes can be added to the steeping water to facilitate the separation of grain components and increase starch recovery. Sulfur dioxide, sodium metabisulfite, sodium bisulfite, or sodium hydrogen sulfite, with variable effective concentrations, are typically added to solubilize the protein matrix enveloping the starch granules in the endosperm (De Mesa-Stonestreet et al. 2010).

Table 2.1 Examples of techniques used for food plant cell disruption

Type of disruption	Procedure	Food	Tissue	Reference
Mechanical homogenization	Colloid milling and homogenization	Rice	Bran	Anderson and Guraya 2001
	Centrifugal grinding and air dehulling	Pea, chickpea, lentil	Seed	Boye et al. 2010
	Cool mortar with lysis buffer	Rice	Embryo	Fukuda et al. 2003
	Cool mortar with Tris–HCl	Olive tree	Seed	Alche et al. 2006
	Wet-milling with sulphur dioxide	Sorghum	Seed	De Mesa-Stonestreet et al. 2010
Ultrasonic homogenization	Acoustic transducer	Soybean, rice	Root, sheath/hypocotyl, leaf	Toorchi et al. 2008
	Ultrasonic generator	Rice	Bran	Chittapalo and Noomhorm 2009
Pressure homogenization	High pressure homogenization	Peanut	Seed	Dong et al. 2011
		Rapeseed	Seed	Barbin et al. 2011
Temperature treatments	Mortar and pestle with liquid N_2	Tomato	Pollen	Sheoran et al. 2009
		Olive	Leaf	Wang et al. 2003
		Apple, banana	Mesocarp/exocarp	Song et al. 2006
		Peanut	Seed	Liang et al. 2006
		Maize	Endosperm	Méchin et al. 2007
		Potato	Tuber	Delaplace et al. 2006
	Pulverization in dry ice and grinding in liquid N_2	Grape	Berry cluster	Vincent et al. 2006
	Microwave, dry heating and parboiling	Rice	Bran	Khan et al. 2011
	Wet-milling with temperature	Sorghum	Seed	De Mesa-Stonestreet et al. 2010
Osmotic and chemical lysis	Hexadecyltrimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS) or isopropylalcohol (IPA)	Lactococcus lactis strains	–	Doolan and Wilkinson 2009

2.2.1.2 Ultrasonic Homogenization

In recent years, some researchers have used the ultrasonic method for protein pellet homogenization. The energy is produced by an acoustic transducer coupled with the pellet in the microtube, which is carried out manually in small batches. Toorchi et al. (2008) have attempted to use a high-performance, single-tube sample preparation device (Covaris) for the noncontact disruption and uniform preparation of three different plant tissues from soybean (root, hypocotyl, and leaf) and rice (root, leaf sheath, and leaf).

Many researchers have investigated the advantages of ultrasonic-assisted extraction compared with the conventional method. Chittapalo and Noomhorm (2009) reported that protein yield increased using ultrasound and that this process can enhance existing extraction processes and enable new commercial extraction opportunities.

2.2.1.3 Pressure Homogenization

The use of high-pressure homogenization (HPH) for the extraction of food proteins has been investigated. Higher pressures (40 and 80 MPa) produced approximately double protein extraction compared to atmospheric pressure. Dong et al. (2011) have suggested that HPH treatment could increase the susceptibility of peanut proteins to proteolytic enzymes such as alcalase. The increase may be related to the denaturation, unfolding, or dissociation of the proteins into monomers, allowing the accessibility of enzyme to the binding sites. HPH revealed no alteration of protein solubility when compared with the raw protein with pH adjusted in rapeseed protein concentrates (Barbin et al. 2011).

2.2.1.4 Temperature Treatments

Temperature treatments include the use of freeze–thaw and heat treatments. Freeze–thawing uses the effect of ice crystal formation in the tissue during the freezing process. Lysis of the cells or tissues is usually achieved by flash-freezing the cells in liquid nitrogen and homogenizing in a mortar with a pestle. Examples of this process are found in the analysis of leaves (Wang et al. 2003), fruits (Song et al. 2006), and seeds (Liang et al. 2006; Méchin et al. 2007). Vincent et al. (2006) developed a very efficient cell disruption method for grape berry clusters, which were pulverized frozen with dry ice using a stainless steel blender.

The use of heat is common in protein processing. Heating protein solutions usually improves their solubility, emulsifying, and foaming properties, but it makes protein extraction more difficult as reported in rice bran (Tang et al. 2002; Khan et al. 2011). Another approach is the application of heat during the wet-milling process. Steeping experiments have been done on temperature and holding time on sorghum grain (De Mesa-Stonestreet et al. 2010).

2.2.1.5 Osmotic and Chemical Lysis

Cell permeabilization or cell lysis can be performed by osmotic shock or chemical treatment. The use of osmotic shock implies cell suspension in a gently shaken hypertonic solution. Chemical treatment can include antibiotics, chelating agents, detergents, and solvents capable of disintegrating the cells. This procedure relies on the selective interaction of the chosen chemicals with components of the membrane and allows proteins to seep through the cell wall. The application of two or more procedures combined with the cell-wall disruption is also reported (Klimek-Ochab et al. 2011). These procedures are used in cell cultures of bacteria, yeast, or fungi. Doolan and Wilkinson (2009) have compared the effects of various chemicals on cell permeability in *Lactococcus lactis* strains with the aim of selectively releasing important intracellular ripening enzymes. Their findings permit a better understanding of methods affecting cell permeability and can allow development of food-grade technologies for protein released from cells.

2.2.2 Protein Solubilization/Precipitation

Protein solubilization is considered one of the key steps in proteomic sample preparation procedures. It is generally employed to separate proteins in the sample selectively from different substances that may interfere in the proteomic assay (Berkelman and Stenstedt 1998). The solubilization/precipitation process strongly affects the quality of the final results and thus determines the success of the entire experiment. Taking into account the immense variety of proteins and the huge number of interfering contaminants present in food-derived extracts, simultaneous solubilization of all proteins remains a great challenge.

Each food sample requires a specific protocol that needs to be optimized to minimize proteolysis and modification of proteins (Bodzon-Kulakowska et al. 2007). For animal tissues, which have higher protein yields, various protein solubilization buffers, including the use of chaotrophic agents, detergents, reducing agents, buffers, and ampholites are used (Pedreschi et al. 2010). The proper use of these additives avoids protein modifications, aggregation, or precipitation that may result in the occurrence of artifacts and the subsequent lowering of protein yield (Gorg et al. 2004).

2.2.2.1 Organic Solvents

The main organic solvents and additives used to extract proteins from food sources are shown in Table 2.2. Many studies performed in the last few years aimed to compare different protein solubilization methods suitable for proteomic analysis (Jiang et al. 2004; Natarajan et al. 2005; He and Wang 2008). The most common

Table 2.2 Examples of organic solvents and additives used to extract proteins from food sources

Solvent (s)	Food	Tissue	Reference
Acetic acid/urea/cetyltrimethylammonium bromide	Rice	Bran	Hamada 1997
Aqueous ethanol	Distiller's grain	Grain	Cookman and Glatz 2009
Aqueous isopropanol	Soybean	Seed	Natarajan et al. 2009
	Rapeseed	Seed	Barbin et al. 2011
Ethanol	*Saccharina japonica*	–	Kim et al. 2011
Glacial acetic acid	Sorghum	–	de Mesa-Stonestreet et al. 2010
Phenol	Tomato	Pollen grain	Sheoran et al. 2009
	Potato	Tuber	Delaplace et al. 2006
	Aloe vera	Leaf	He and Wang 2008
	Soybean	Seed	Natarajan et al. 2005
Phenol/ammonium acetate	Barley	Root	Hurkman and Tanaka 1986
	Avocado/tomato/orange/banana	Fruit	Saravanan and Rose 2004
	Banana	Leaf	Carpentier et al. 2007
	Grape	Fruit	Vincent et al. 2006
	Pear	Fruit	Pedreschi et al. 2007
	Apple/strawberry	Fruit	Zheng et al. 2007
Phenol/methanol-ammonium acetate	Coniferous	Seed	Zhen and Shi 2011
	Banana/apple/potato	Tissues	Carpentier et al. 2005
Sodium dodecyl sulphate/acetone	Coniferous	Seed	Zhen and Shi 2011
	Potato	Tuber	Delaplace et al. 2006
Sodium dodecyl sulphate/TCA/acetone	Apple/banana	Tissue	Song et al. 2006
TCA	Bean	Anther	Wu and Wang 1984
TCA/acetone	Citrus	Leaf	Maserti et al. 2007
	Soybean	Seed	Natarajan et al. 2006
	Soybean	Leaf	Xu et al. 2006
	Coniferous	Seed	Zhen and Shi 2011
	Tomato	Pollen grain	Sheoran et al. 2009
	Aloe vera	Leaf	He and Wang 2008
	Apple/banana	Tissues	Song et al. 2006
TCA/acetone/phenol	Olive	Leaf	Wang et al. 2003
	Bamboo/grape/lemon	Leaf	Wang et al. 2006
	Apple/orange/tomato	Fruit	Wang et al. 2006
Thiourea/urea	Soybean	Seed	Natarajan et al. 2005
	Apple/banana	Tissues	Song et al. 2006
Tris–HCl buffer	Tomato	Pollen grain	Sheoran et al. 2009

method used for the extraction of plant proteins is tricholoracetic acid (TCA)/acetone precipitation as proposed by Damerval et al. (1986). This method has been used to extract proteins from different tissues of cereals, legumes, and fruits. The extreme pH

and negative charge of TCA and the addition of acetone realizes an immediate denaturation of the protein, along with precipitation, thereby instantly arresting the activity of proteolytic and other modifying enzymes. However, a disadvantage of TCA-precipitated proteins is that they are difficult to redissolve (Nandakumar et al. 2003). Sample solubility can be improved by using an appropriate mixture of chaotropic agents (urea or thiourea), and new efficient detergents (such as sodium dodecyl sulphate, SDS). In the last decade, the phenol extraction procedure has been widely used because of its high clean-up capacity. In contrast to its strong solvent action on proteins, phenol has little predisposition to dissolve polysaccharides and nucleic acids. However, phenol shows the disadvantages of being more time consuming than other sample precipitation procedures and of being toxic.

The alcoholic extraction process after dehulling and conventional deoiling has a high efficiency of protein recovery. Aqueous alcohols (ethanol, isopropyl alcohol, butanol) are widely used on a commercial scale to remove phenolics, oligosaccharides, or inhibitors from defatted meals and seeds (Moure et al. 2006). However, as a result of the extraction with these alcoholic solvents, protein structures can be coagulated and therefore show reduced functional properties. To avoid these problems and to obtain protein concentrates or isolates with good functionality and suitable as food ingredients, mechanical and thermal treatments are applied (Moure et al. 2006; Barbin et al. 2011). Recently, extractions with different organic solvents, such as n-hexane, 2-methyl pentane, diethyl ether, acetone, 2-propanol, and ethanol were compared regarding effectiveness, suitability, and protein solubility of the full-fat and defatted lupin (Bader et al. 2011).

2.2.2.2 Aqueous Solutions

In recent years, because of the growing environmental concerns over the use of organic solvents to extract oil/protein from oil-bearing food materials, aqueous extraction is gaining attention. Water is also operationally advantageous over alcohols because it is nonflammable and neither explosive nor toxic. Commercially, the production of protein concentrates (48–70% protein) or isolates (85–90% protein) consists of an aqueous solubilization of protein and carbohydrates at acid, neutral, or alkaline pH and the selective recovery of the solubilized protein, separation, and, optionally, washing and neutralization before drying. The protein extraction yield and properties are influenced by the type of extraction process and by different factors such as pH, salts concentration, the ionic strength of the medium, net charge, and electrostatic repulsions (Tan et al. 2011).

A number of acid and alkaline protein extraction protocols have been published from various plant and animal tissues. In the last decade, different studies have focused on evaluating the effect of extraction methods on the functional and rheological properties of proteins recovered from by-products of the meat and fish industry (Liang and Hultin 2003; Chaijan et al. 2006; Hrynets et al. 2010, 2011; Moayedi et al. 2010; Omana et al. 2010). In the case of plant proteins, the ideal extraction method is particularly challenging due to the metabolic and structural characteristics

of plant tissues, including the cell wall matrix. The majority of alkaline extraction protocols are based on the so-called Osborne method (Osborne 1924), but each method is optimized according to the aim of the study and the type of vegetal protein source. Recent studies report the use of mainly sodium and calcium salts to extract proteins from different vegetal foods (Ghaly and Alkoaik 2010; Horax et al. 2010; Lestari et al. 2010; Karaca et al. 2011; Nadal et al. 2011). These extraction methods are simple because the agents required are easily available. However, as a result of the degradation at high pH conditions, the protein yield is generally low. Also, the protein quality can be altered by alkaline processing due to undesirable reactions involving racemization of amino acids, formation of toxic compounds such as lysinoalanine, reduction of digestibility, loss of essential amino acids, and decrease in nutritive value. Furthermore, the remaining alkali needs to be washed thoroughly from the final product, leading to generation of a large amount of wastewater (Sereewatthanawut et al. 2008). To optimize protein precipitation recovery different strategies have been developed. Use of additives, such as TCA or carboxymethylcellulose is generally accepted (Massoura et al. 1998). Extraction and further formation of protein micelles have also been proposed (Krause et al. 2002; Murray 2003; Ser et al. 2008; Green et al. 2010). This method has been demonstrated to reduce the concentration of problematic antinutritional or toxic factors, including the glucosinolates and their degradation products during canola protein extraction (Tan et al. 2011).

2.2.2.3 Aqueous Enzymatic Extraction

An alternative approach combining aqueous and enzymatic extraction is attracting attention. Studies using this extraction process are shown in Table 2.3. Enzymes can aid in the extraction of proteins in several ways. Carbohydrases, which can attack the cell wall components, may increase protein yield by liberating more protein from the matrix source (Ansharullah et al. 1997; Wang et al. 1999; Tang et al. 2002). A combination of cell wall-hydrolyzing enzymes (i.e., Viscozyme L) has been used to cleave linkages within the polysaccharide matrix effectively and hence, liberate more intracellular protein from oat bran (Guan and Yao 2008). In the last few years, different proteases, alone or in combination, have been used to partially hydrolyze proteins to peptides, increasing their solubility and making them more easily extractable. Recently, De Moura and co-workers (2011) developed a two-stage countercurrent aqueous enzymatic extraction process for soybean, significantly reducing the amount of water used. They achieved slightly higher oil and protein extraction yields than those from standard single-stage aqueous enzymatic extraction.

Aqueous enzymatic protein extraction has been defined as an environmentally friendly, safe, and cheap alternative to extract oil and protein simultaneously (Latif and Anwar 2009). Moreover, this process avoids serious damage to the proteins produced by the refining steps, improving their nutritional and functional properties (Domínguez et al. 1994; Moure et al. 2000). However, and although the enzymatic extraction process produces no toxic chemicals, it shows some disadvantages, such

Table 2.3 Aqueous-enzymatic extraction processes used to obtain protein isolates or concentrates from food sources

Enzyme	Food	Tissue/sample	Protein extracted (%)	Reference
Alcalase™	Rice	Bran	81.0	Hamada 2000
Alcalase 2.4 L	Rapeseed	Seed	66.7	Zhang et al. 2007
	Peanut	Seed	82.5	Wang et al. 2008b
	Peanut	Roasted seed	80.1	Zhang et al. 2011
Alcalase + Protamex (1:3)	Tea	Leave pulp	47.8	Shen et al. 2008
Alkaline protease	Rice	Broken rice	75.5	Hou et al. 2010
Flavourzyme	Rice	Bran	88.0	Hamada 2000
Glucoamylase	Lentil	Bean	–	Bildstein et al. 2008
Neutrase 1.5MG	Coconut	Meat	83.0	Sant'Anna et al. 2003
Olivex + Celluclast	*Guevina avellana*	Pressed cakes	85.8	Moure et al. 2002
Papain	Rice	Broken rice	46.3	Hou et al. 2010
Pectinase + Protease P	Rice	Bran	80.0	Tang et al. 2003
Phytase	Rice	Bran	80.0	Wang et al. 1999
Protex 6 L	Distiller's grain	Grain	90.0	Cookman and Glatz 2009
	Lupin	Seed	77.2	Jung 2009
	Soybean	Seed	84.6	Jung 2009
	Soybean	Flakes	96.0	De Moura et al. 2011
Protex 7 L	Sesame	Seed	87.1	Latif and Anwar 2011
	Moringa oleifera	Seed	75.4	Latif and Anwar 2009
Viscozyme L	Coconut	Meat	83.0	Sant'Anna et al. 2003
	Oat	Bran	56.2	Guan and Yao 2008
Viscozyme L + Cellulast 1.5 L	Rice	Bran	37.0	Tang et al. 2002
		Bran	53.0	Ansharullah et al. 1997
Xylanase	Rice	Bran	82.0	Wang et al. 1999
Xylanase + Phytase amylase	Rice	Bran	75.0	Wang et al. 1999

as the long time required and the high cost of enzymes that makes this strategy uneconomical. The use of immobilized enzyme in protein extraction may reduce the overall cost by allowing the reuse of enzymes.

2.2.2.4 Subcritical Water

Recent studies demonstrate the use of water at subcritical conditions as an environmentally friendly reaction medium to extract proteins from different food sources. Subcritical water is water that maintains its liquid state in the temperature range of 100–374°C under pressurized conditions. Its unique properties, such as a lower relative dielectric constant and a higher ion product than ambient water, make subcritical water a promising extraction solvent for various compounds, including proteins (Hata et al. 2008). Ho et al. (2007) used pressurized low-polarity water to extract proteins from defatted flaxseed meal. A number of studies have demonstrated the ability of water at subcritical conditions to extract proteins from rice bran and soybean meal with high protein yields and good functional properties (Watchararuji et al. 2008; Fabian and Ju 2011).

2.2.3 Protein Enrichments Methods

Once the protein fraction has been isolated from the rest of the constituents and the interference substances have been eliminated, there are still some other steps that are needed prior to the analysis of the sample by mass spectrometry (MS). Despite the last technological developments, no single analytical method exists covering the protein concentration range present in a specific sample. Sometimes the total protein content is very low or the objective is the determination of minor proteins with post-translational modifications (e.g., phosphorylation).

In many cases the methods described in this part are comparable (if not the same) to those previously seen in this chapter during protein extraction. However, this section is focused more on those steps to be applied once the protein fraction has been separated from other interfering components. As a matter of fact, the purpose of fractionation and enrichment methods is to obtain distinguishable fractions and increase the concentration of the proteins of interest.

2.2.3.1 Centrifugation

The use of centrifugation is one of the simplest methods used for isolation and enrichment/fractionation of proteins. Centrifugation can be used for different purposes. It can be a first step to separate different cell substructures where our proteins of interest are locally concentrated, for instance, mitochondria, membrane, or nucleus. This process involves multiple centrifugation steps and, as a result, the

cellular homogenate is separated into different layers based on the molecular weight, size, and shape of each component. Afterwards, solubilization steps, as explained above, and enrichment and fractionation steps should be carried out to isolate the protein fraction from the selected layer prior to MS analysis.

Apart from its use separating crude mixtures of cell components, centrifugation is also commonly used to fractionate a protein mixture into different fractions. The separation takes place based on the coefficient of sedimentation of the proteins. This coefficient is usually expressed in *Svedberg units* (S), and the smaller the S value, the slower a molecule moves in a centrifugal field. Separation will depend on the mass, the shape, and the protein density. Numerous examples are found in the literature using the differential coefficient of sedimentation of the proteins to carry out fractionation (Sharma et al. 2010; Jiang et al. 2011). The efficiency of this fractionation step can be enhanced using gradient centrifugation, where the centrifuge tube is filled with a solution of sucrose, forming a density gradient.

2.2.3.2 Precipitation

It is recognized that among the different precipitants the most widespread is ammonium sulphate (Bodzon-Kulakowska et al. 2007). The addition of high amounts of this salt or other such as sodium chloride into a protein solution provokes an increase of protein interactions followed by protein aggregation and finally precipitation. This is known as a salting-out process and, as the salt concentration needed for protein precipitation varies from one protein to another, it allows selective protein separation. An alternative salting-out method using decreasing solutions of salt can also be used to enrich previously precipitated protein fractions. This salting-out approach has been used to separate the main storage soybean proteins, glycinin and β-conglycinin (Deak et al. 2006).

Another type of protein enrichment is immunoprecipitation, based upon the binding of the antigen to its specific antibody to form the antigen–antibody complex. In general it offers high recoveries of the proteins and it is widely used for food allergens (Pastorello and Trambaioli 2001).

2.2.3.3 Electrophoretic Methods

Electrophoresis separates mixtures of proteins based on charge, charge/mass ratio, size, or shape. This technique is mainly used as an analytical and preparative tool, especially one-dimensional separation, often employed as a pre-fractionating technique (Guttman et al. 2004; Jorgenson and Evans 2004). Often, laboratories dedicate one-dimensional gel electrophoresis (1DE) to evaluate the outcome of protein purification preceding the analysis by (2DE) (Chen et al. 2007).

Electrophoretic pre-fractionation methods include electrokinetic methodologies performed in free solution, essentially all relying on isoelectric focusing (IEF) steps. Purification using IEF is especially advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins remain in solution in their native conformation. Based on the IEF principle, different instruments have been developed such as the Rotofor, a multicompartmental device with focusing chambers that allows the fractionation of volumes of sample (12–60 mL) containing micrograms to grams of protein (Hey et al. 2008). Another well-known device is the so-called "Off-Gel IEF" (Keidel et al. 2011). Upon application of an electric field perpendicularly to the liquid chamber, the current lines penetrate into the chamber and extract charged proteins from the solution into the IEF gel. In its multicompartment format, the protein fractions are separated by ranges of pI depending on their positioning over the IEF gel strip. Other instruments of interest are the Octopus, a continuous-flow device for isoelectric focusing in an upward flowing liquid curtain, and the Gradiflow, where different pI cuts are obtained by a multistep passage through two compartments buffered at different pH values (Righetti et al. 2003).

Depending on the complexity of the samples, the separated fractions can be analyzed directly by MS or in some cases they may undergo a subsequent separation step in a second dimension, generally SDS-PAGE, to separate the proteins according to their molecular weight. In the first case, the possible presence of ampholytes may imply an extra step to remove them and avoid disturbance in MS.

2.2.3.4 Chromatographic Methods

Liquid chromatography (LC) techniques are the most commonly used in proteome pre-fractionation prior to in-depth analysis. The separation of the different proteins is achieved according to their charge, hydrophobicity, size, or specificity. In some cases, chromatographic methods can also be used to eliminate some interference substances (e.g., salts) coming from previous enrichment steps.

Among LC fractionation methods, ion-exchange chromatography (IEX) is probably the most used, with proteins being separated according to their pI. Acidic proteins are usually fractionated by anion-exchange chromatography whereas basic proteins are fractionated by cation-exchange chromatography. IEX has been often used to separate milk proteins as reported by Gómez-Ruiz et al. (2007a), who used cation-exchange chromatography to separate sheep milk caseins.

Reverse phase LC (RP-LC) separates proteins according to their hydrophobicity. Proteins are adsorbed on a stationary phase carrying hydrophobic groups, and are eluted with increasing concentration of an organic solvent, generally acetonitrile. RP-LC is widely used in proteomics in combination with IEX and MS analysis, usually in shotgun multidimensional strategies that are used as an alternative to 2-D-PAGE technology. A special case of chromatography based on hydrophobic interactions uses a high concentration of lyotropic salts (frequently ammonium sulphate) to expose the hydrophobic parts of proteins towards the hydrophobic patches of

solid-phase sorbents. Desorption is promoted by using a decreasing concentration of the lyotropic salts.

Pre-fractionation with chromatographic methods is also used to investigate post-translational modifications such as glycosylation or phosphorylation, to cite perhaps the two most important. These modifications are mainly studied using affinity chromatography (AC). This chromatography utilizes highly specific biological interactions (i.e., antigen–antibody, receptor–ligand, enzyme–substrate/inhibitor, etc.). AC results are quite adequate for accessing low concentrated proteins in complex samples, in some cases through the depletion of high abundance proteins that remain bound to the column. Examples of AC are heparin chromatography, broadly used for studying microbial proteins, or lectin chromatography that is specially used for glycoproteins (Lee and Lee 2004; Azarkan et al. 2007). Immobilized metal affinity chromatography (IMAC) is used to enrich phosphoproteins. This chromatography is based on formation of coordinate bonds between basic groups on protein surface and metal ions. The major drawback is that little or no binding to Fe(III) or Ga(III) charged resins is observed at neutral pH, and using low-pH buffers may provoke protein denaturalization or precipitation in the column (Schmidt et al. 2007).

Some other techniques such as size-exclusion chromatography (SEC) separate proteins according to their molecular mass, as the second dimension of 2-D-PAGE. However, unlike 2-D-PAGE this chromatography can be used under nondenaturing conditions allowing the study of protein complexes. As an example, SEC has been used for the evaluation of the bread-making quality of hard spring wheat flours (Ohm et al. 2009). Additional methods based on the use of chip-based arrays are gaining importance recently, with surface-enhanced laser desorption/ionization (SELDI) one of their maximum exponents (Righetti et al. 2005). Unlike chromatography separation, here only retained proteins are eventually studied and the other proteins are removed by one or more washing steps. Subsequently, the use of a pulse of laser light provokes the desorption of the proteins of interest which are converted into gaseous ions and analyzed by MS, typically using time of flight (TOF) analyzers.

2.3 Protein Digestion

Once the proteins have been isolated from interfering compounds (other food components such as lipids, nucleic acids, phenolic compounds, or carbohydrates) they are usually analyzed by 1D or 2-D SDS-PAGE, depending of the complexity of the sample. Gel electrophoresis analysis is typically followed by protein digestion, a key procedure prior to the identification of proteins by MS. However, in some cases digestion is carried out without electrophoretic separation. For instance, direct digestion of a mixture of proteins is adequate when a broad survey of the identifiable protein components is desired or to minimize the loss of peptides by binding to the polyacrylamide when characterizing post-translational modifications (Kinter and Sherman 2005).

Different proteolytic agents are used for protein digestion, including enzymes such as trypsin, different endoproteases (Lys-C, Arg-C, Asp-N, Glu-C), or chymotrypsin, as well as chemical reagents such as hydroxylamine or cyanogens bromide. The specificity of the amide bond or bonds cleaved by these reagents allows the obtaining of specific peptides that facilitate the interpretation of their mass spectra and database search. Trypsin is certainly the most popular reagent because it shows many advantages compared to other enzymes and chemical reagents, in addition to its relatively low cost of production and high purity. This enzyme cleaves amide bonds at the C-terminal side of Lys and Arg residues except when these bonds are to Pro. Apart from this selectivity, Arg and Lys are common amino acids distributed through most proteomes such that tryptic cleavage yields peptides with an average length suitable for MS. Finally, trypsin cleavage yields peptides containing a strongly basic residue (Lys or Arg) at the C-terminal, a fact that facilitates the interpretation of collision-induced dissociation (CID) mass spectra (Couto et al. 2011).

When talking about protein digestion in proteomic studies we mainly think of two types of digestion: "in-gel" digestion and "in-solution" digestion. Most proteomic studies perform in-gel digestion of proteins previously separated by their charge and/or their molecular weight (1D or 2-D SDS-PAGE). Identification of proteins from polyacrylamide gels offers a number of important advantages compared to gel-free approaches, such as higher dynamic range of analysis of protein mixtures (ratio of lowest to highest abundance protein detectable) or removal of low molecular weight impurities before the MS analysis. In-gel protein digestion was first established by Rosenfeld et al. (1992). The typical steps of the method have remained the same since then, although small variations have been introduced to improve its performance. Destaining, reduction, and alkylation of Cys, enzymatic cleavage of proteins into peptides, and extraction of peptides from the gel are described as essential steps in obtaining high-quality mass spectra. Analysis by electrospray (ESI)-MS, less tolerant to salts, requires an additional desalting step which is optional for MALDI-MS (Granvogl et al. 2007)

Despite its widespread use, conventional tryptic digestion is very time consuming, with a typical digestion time in the range of several hours to half a day (Park and Russell 2000). This is a clear limitation to the production of high-throughputs in proteomic analysis. Therefore, in recent times many efforts have been focused on developing efficient and fast protein digestion methods. Several alternatives to the standard protocol have been proposed, many of them based on the use of electromagnetic waves (i.e., electromagnetic radiation), such as microwaves, infrared (IR) radiation, and ultraviolet (UV) light to accelerate protein digestion (Chen et al. 2011; Dycka et al. 2012). Among these strategies, the use of IR-assisted digestion seems to be the most promising approach due to its safety compared to the other electromagnetic waves. Other alternatives are the use of modified trypsin, for instance, by reductive methylation which decreases autolysis and shifts its optimal catalytic temperature to 50–60°C. This modified trypsin allows the reduction of digestion times from 16 h to 30 min without losing efficiency. For in-solution digestion, immobilized trypsin systems as part of a microchip bioreactor offer a

very efficient alternative to conventional methods. As an example, the use of a fiber-based microchip bioreactor provides on-chip digestion in less than 5 s with similar tryptic digests to those obtained by the conventional in-solution tryptic digestion (Fan and Chen 2007).

During each step along the protein digestion experiments extreme care is necessary to avoid contamination that can compromise MS analyses and the outcome of the study. In some cases the contamination refers to low molecular weight compounds (from either the polyacrylamide gel or the subsequent digestion of specific bands) that will not provoke erroneous protein identification but will complicate peptide detection due to higher noise levels. The use of high-purity reagents throughout the experiment, especially for gel-electrophoresis and digestion can significantly minimize this type of contamination. On other occasions, contamination with keratins, proteins derived from skin and hair, is the main problem. In this case wearing gloves and an adequate handling of the laboratory consumables (e.g., pipette tips) and reagents will limit this contamination.

2.4 Food Peptide Extraction and Fractionation

Generally, food peptide content is not as abundant as would be desirable. In addition to this, the presence of nonpeptidic constituents (i.e., lipids, sugars) may also interfere in peptide analysis. Therefore, in practice it is difficult to analyze food peptides with good accuracy without performing a sample preparation step. This sample preparation can comprise diverse procedures for isolation, purification, and preconcentration of the analyte, more than one step being required in many cases (Poliwoda and Wieczorek 2009).

RP-LC and capillary electrophoresis (CE), are the basic analytical methods used for chemometrical analysis of food peptidome (Minkiewicz et al. 2008). In relation to CE and capillary electrochromatography (CEC), restrictions come from the small sample volume applied (nano- to picolitre) that necessitates the application of pre-concentration and pre-separation steps in samples with low peptide concentration or complex mixtures (Kasicka 2012).

In general, food samples are first subjected to a preliminary sample cleanup step to remove interfering substances and then, different fractionation steps are applied, as has been widely revised (González de Llano et al. 2004; Asensio-Ramos et al. 2009; Hernández-Ledesma et al. 2012). Several options that are summarized in Table 2.4 may be taken. Direct peptide analysis on food samples without any preparation treatment is not often reported in the literature (Cheison et al. 2010).

Peptide derivatization may be necessary in some analyses for better detection (Wang et al. 2011). Most derivatizations are developed with fluorescent labels to become detectable with fluorescence detection whose limit of detections (LODs) is about two to three orders of magnitude lower than common UV-absorption detections (Kasicka 2012). An example may be found in the determination of glutathione in must and white wine during alcoholic fermentation (Lavigne et al. 2007).

2.4.1 Extraction and Preliminary Sample Cleanup

Hydrophilic peptides are generally extracted with homogenization in water or in solutions of organic acids whereas organic solvents are used to obtain highly hydrophobic peptides. Homogenization in a mixture of organic solvents (chloroform/methanol) can be used for peptide extraction as well as for the removal of sample interferences after producing a biphasic system. By using this method, Kostyra et al. (2003) studied the opioid activity of cheese and fermented milk samples, On the other hand, homogenization in water has been widely applied on cheese, fish, meat, and cereals samples as shown in Table 2.4. Typically, the ratio of water to cheese used was 2:1 in the homogenization process, followed by an incubation step of an hour at 60°C (Gómez-Ruiz et al. 2002; Meyer et al. 2009).

Peptide extraction is usually followed by a preliminary sample cleanup for removal of other food components (i.e., proteins, lipids). Deproteinization, the most important preliminary cleanup procedure in peptide analysis, is carried out by precipitation of protein using several agents. Deproteinization could also act as a fractionation procedure for peptides because their solubility depends on the precipitant agent and its proportion (Cheng et al. 2010a,b). After precipitation, centrifugation and filtration methods are used to separate proteins from soluble peptides. In addition, the application of heat treatments or ultracentrifugation steps at high speed to eliminate the proteins has been reported (Gómez-Ruiz et al. 2007b; Ho et al. 2010).

The selectivity of precipitation directly depends on the type of precipitating agent applied. In addition to the use of organic solvents such as ethanol, methanol, or acetone, solutions containing acids such as TCA or trifluoroacetic acid (TFA) are classical protein precipitants (Juan-García et al. 2009; Escudero et al. 2010). Salting-out precipitation, based on polarity, with high concentrations of salts or precipitation by adjusting the pH to the pI of protein (Contreras et al. 2010; Pihlanto et al. 2010) are other options. A representative example is found in the isolation and identification of an angiotensin I-converting enzyme (ACE) inhibitory peptide from whole buckwheat seeds after consecutive cleanup steps of diethyl ether extraction in order to remove most of the fat content and deproteinization by adjusting the pH to the pI of buckwheat protein (Ma et al. 2006).

In some cases, the application of homogenization and/or deproteinization is enough to proceed with peptide analysis (Contreras et al. 2010). Unfortunately, most of the samples need additional steps to achieve suitable peptide isolation and concentration levels before the analysis.

2.4.2 Fractionation

2.4.2.1 Ultrafiltration

Ultrafiltration is mainly useful for fractionating peptides as well as the removal of proteins and other macromolecules based on their molecular size. Dedicated

Table 2.4 Examples of food sample preparation for peptidomic analysis

Food matrix	Extraction and clean-up	Fractionation techniques	References
Dairy products			
Cheese, milk, yoghurt and infant formula	Homogenization, centrifugation and deproteinization by pH adjustment	Ultrafiltration	De Noni and Cattaneo 2010
Dry-off cows milk	Centrifugation and ultracentrifugation	Ultrafiltration	Ho et al. 2010
Fermented milk	Centrifugation	SPE	Hernández-Ledesma et al. 2005
Manchego cheese	Homogenization, centrifugation, filtration and ultracentrifugation	Ultrafiltration and SEC	Taborda et al. 2007; Gómez-Ruiz et al. 2007b
Whey protein concentrate hydrolysate	Centrifugation and filtration	Ultrafiltration	Contreras et al. 2011
Fish and meat			
Rainbow trout muscle	Homogenization and centrifugation	Ultrafiltration and SPE Derivatization of anserine	Bauchart et al. 2007
Cuttlefish protein hydrolysate	Centrifugation	SEC	Balti et al. 2010
Loach protein hydrolysate	Centrifugation	Ultrafiltration, IEX and SEC	You et al. 2010
Dry-cured ham	Homogenization, centrifugation, filtration and deproteinization by ethanol addition	SEC	Mora et al. 2010, 2011
Pork meat digest	Centrifugation and deproteinization by ethanol addition.	–	Escudero et al. 2010
Eggs			
Egg white protein hydrolysate	Centrifugation	SEC and IEX	Liu et al. 2010
Egg white protein hydrolysate	Centrifugation	Ultrafiltration	Miguel et al. 2004
Drinks			
Beer	–	SPE and SEC	Picariello et al. 2011
Must and wines	Centrifugation (not bottled wines)	Derivatization of SH functions	Lavigne et al. 2007
White and red wines	Centrifugation	Ultrafiltration and SEC	Pozo-Bayón et al. 2007

Vegetable foods			
Corn zein hydrolysate	Pigments extraction and centrifugation	Ultrafiltration	Tang et al. 2010
Fermented soybean extract	Filtration and dialysis	Ultrafiltration and IEX	Rho et al. 2009
Soy protein hydrolysate product	pH adjustment and centrifugation	–	Johns et al. 2011
Pea protein hydrolysate	Centrifugation	Ultrafiltration and SPE	Li and Aluko 2010
Potato protein hydrolysate	Salting-out precipitation and centrifugation	SEC	Cheng et al. 2010a, b

IEX ion exchange chromatography, *SEC* size exclusion chromatography, *SPE* solid phase extraction

membranes are mostly made of polysulfone or cellulose derivatives. Cellulose membranes have excellent hydrophilicity, which is very important in minimizing fouling, but they possess low chemical resistance and poor mechanical strength. However, polysulfone membranes provide high rigidity but foul earlier because of their hydrophobicity (Doyen et al. 2011). Commercially, membranes offer a wide range of cutoffs (500–100 kDa) and different formats including centrifugal units or cassettes for peristaltic lab systems. Fractionation of peptides has been achieved in food samples by applying ultrafiltration with more than one cutoff membrane. As an example, Samaranayaka et al. (2010) searched the presence of antioxidant and ACE inhibitory peptides in a hake protein hydrolyzate using an ultrafiltration unit with different molecular mass cutoff membranes (10, 3, and 1 kDa). In summary, ultrafiltration presents some advantages as the sample is not diluted or organic solvents are not required. Therefore, in some cases after the ultrafiltration step no additional fractionation processes are applied before analysis, such as in cheese (Bütikofer et al. 2008) or champagne wine samples (Person et al. 2004). Nevertheless, samples often need further pre-treatment procedures that mainly improve the analyte concentration.

A recent technology named electrodialysis with ultrafiltration membranes (EDUF) has been developed to fractionate peptides from complex mixtures on the basis of their electrical charge, size, or molecular weight. A conventional electrodialysis is used but some ion exchange membranes are replaced by ultrafiltration ones. This equipment has been employed for the concentration and selective separation of bioactive peptides from an alfalfa white protein hydrolyzate (Firdaous et al. 2009). A successful use of these membranes has also been reported, isolating an antihypertensive peptide from a tryptic hydrolyzate of β-lactoglobulin (Poulin et al. 2007).

2.4.2.2 Low-Pressure Liquid Chromatography

Low-pressure size exclusion chromatography (SEC) fractionates peptides on the basis of their molecular size. This technique separates analytes through a bed of porous beads where they can either enter or be excluded from the internal space of the beads based on their size. Elution occurs from the largest to the smallest analyte over time (Ly and Wasinger 2011). Several resins with different pore sizes are commercially available. Cross-linked dextran (Sephadex) resins are mostly used but polyacrylamide (BioGel P) or divinylbenzene polymers are also available as stationary phases (Poliwoda and Wieczorek 2009). Depending on the resin composition, peptides are eluted with water, organic acids, ammonia, or ammonium salts, even as alcoholic solutions that reduce potential hydrophobic interactions. For instance, Mora et al. (2011) applied SEC to fractionate peptides released during dry-cured ham processing in a Sephadex G25 column under isocratic conditions in 0.01 N HCl. Other uses have been reported, for instance, to identify ACE inhibitory peptides in white and red wines (Pozo-Bayón et al. 2007).

Low-pressure ion exchange chromatography (IEX) constitutes another technique for peptide fractionation in food analysis. In this case, peptides are fractionated according to their net surface charge/polarity. Porous or nonporous matrices with hydrophilic materials such as cellulose, cross-linked dextrans, polystyrene polymers (Dowex resins), or Bio-Rex membranes are very useful as anion or cation exchange stationary phases. These matrices are substituted with functional groups that determine the charge of the medium (e.g., quaternary ammonium, diethylaminoethyl, sulfopropyl, carboxymethyl, etc.). The ionic strength increases as the elution method can carry a large amount of salts in the elution buffer that makes samples incompatible with techniques such as MS (Ly and Wasinger 2011).

Off-line combination of IEX and SEC has been reported in some food peptide analyses. A representative example is found in the work of Liu et al. (2010), who fractionated an egg white protein hydrolyzate by SEC with Sephadex G-25 resin followed by IEX (Sephadex C-25 column) of those fractions with the highest ACE inhibitory activity. A similar fractionation strategy has also been used in the study of antioxidant peptides in a fish protein hydrolyzate (You et al. 2010), and for the evaluation of the peptide contribution to the umami taste of soy sauces (Lioe et al. 2006).

2.4.2.3 Solid-Phase Extraction

Solid-phase extraction (SPE) is based on the same principle of affinity-based separation as liquid chromatography. SPE enables retention and elution of analytes from complex mixtures, removal of interfering compounds, and sample concentration. SPE is available in normal phase, reverse-phase, and ion exchange modes, reversed-phase being one of the most used formats (Kole et al. 2011). Based on the wide range of physicochemical properties of the analytes, several commercial sorbents (e.g., C_{18}, C_8, C_2, phenyl, cyanopropyl, and ion exchange bonded materials, among others) are supplied to improve the versatility of SPE. Regarding this, in the peptidomic characterization of beer, Picariello et al., (2011) applied the samples directly onto the C_{18} pre-packed cartridges and eluted with acetonitrile/TFA to RP-LC. In other examples, Hernández-Ledesma et al. (2005) treated the water-soluble extract of fermented milk with a Sep-Pak C_{18} cartridge and acetonitrile elution, and a similar extraction step was used by Muguruma et al. (2009) to desalt SEC-eluted fractions from porcine myosin B.

Based on similar principles of SPE techniques, innovative size-reduced devices have recently appeared for concentration, purification, and desalting of peptides prior to analysis by MS. These devices support a membrane or microcolumn that can be of diverse nature (polar, nonpolar, and ion exchange) and feature an optimized procedure for sample preparation. For instance, in the study of trout peptidome changes during storage, Bauchart et al. (2007) used a C_{18} membrane device prior to MALDI-TOF analysis with the aim of removing the perchloric acid used in the previous extraction. Another case is found in the study of beer peptidome in which

residual interfering sugars are removed by applying the sample on C_{18} Zip-Tip microcolumns and peptides eluted with acetonitrile/TFA (Picariello et al. 2011).

2.5 Future Perspectives

Current efforts are mainly focused on the improvement and development of automated systems as today sample preparation implies several labor-intensive and time-consuming handling steps. Despite the generalized use of 2-D electrophoresis, this technology has limitations mainly when dealing with proteins at varying expression levels. An alternative could be the use of automated pre-fractionation methods such as electrokinetic methodologies performed in free solution combined with one-dimensional PAGE and capillary LC-MS/MS. Recent technologies such as SELDI also imply minimal requirements for purification and separation of proteins prior to their analysis by MS. For the investigation of post-translationally modified proteins the future approach seems to be the combined use of affinity-based enrichment and extraction methods and multidimensional separation technologies prior to MS analysis. A persisting challenge is still the development of appropriate enrichment/fractionation techniques to facilitate MS analysis of membrane proteins.

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Acute and Repeated Dose (28 days) Oral Safety Studies of ALIBIRD in Rats

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Abstract

The test substance ALIBIRD formed by three components: oligosaccharides derived from lactulose, a hydrolysate of a whey protein concentrate and a supercritical extract of rosemary in a proportion 1:0.5:0.05 has been prepared in the laboratory. In order to use ALIBIRD as a multifunctional food additive, an evaluation of oral toxicity (acute and 28 days repeated dose) in Wistar rats has been carried out. The ALIBIRD administered in a single oral gavage dose of 2000 mg/kg body weight resulted in no adverse events or mortality. The ALIBIRD product administered as a daily dose of 2000 mg/kg body weight for 28 days by gavage also resulted no adverse effects or mortality. For both studies, no abnormal clinical signs, behavioral changes, body weight changes, or change in food and water consumption occurred. There were no changes in hematological and serum chemistry values, organ weights, or gross or histological characteristics. Taken together the results obtained, it is concluded that the ALIBIRD product is well tolerated in rats at an acute and subchronic (28 days) dose of 2000 mg/kg body weight.

The Institute of Food Science Research (CIAL) has developed the synthesis of a new prebiotic derived from lactulose (OsLu), the preparation of a hydrolysate of whey protein concentrate (WPC) as well as the procedure to obtain extracts of rosemary through supercritical fluid technology. These three complementary food additives have been mixed by weight in a proportion 1:0.5:0.05 respectively under the name ALIBIRD to be used as a multifunctional food additive. Oligosaccharides derived from lactulose (OsLu) are new bioactive carbohydrates synthesized with the aim of obtaining a group of more slowly fermenting prebiotics able to reach distal part of colon without being hydrolysed (*7,9,11,23*). It is known that lactulose stimulates bifidobacteria present in the gastrointestinal tract (*30*) because is resistant to hydrolysis by human intestinal enzymes. It reaches the proximal colon unaltered where it is selectively metabolized by bifidobacteria and lactobacilli and only a reduced percentage of lactulose may reach the distal colon where the potential for disease and disorder is particularly high. Therefore, our studies were focused to produce oligosaccharides able to act in the lower gut. Different *in vitro* fermentation assays using pure cultures (*8*) as well as faecal inocula (*10*) have demonstrated the stimulation of bifidobacteria and lactobacilli growth when these oligosaccharides were used as sole carbon source. When long incubation periods were used in fermentative assays, oligosaccharides derived from lactulose showed higher prebiotic index values than the native disaccharide. Oligosaccharides derived from lactulose may constitute a good alternative to lactulose, and they could belong to a new generation of prebiotics to be used as a functional ingredient for improving the composition of gut microflora.

Dietary proteins are also a source of bioactive components. Food proteins can release a large variety of peptides during *in vivo* digestion as well as during *in vitro* enzymatic hydrolysis. Some of these peptides are structurally similar to endogenous peptides that act as hormones, neurotransmitters or antibiotics and therefore, certain food-derived peptides can

interact with body receptors or enzymes and exert an agonistic or antagonistic effect. Milk proteins are the major source of bioactive peptides that have been reported in recent years (*24*). Whey proteins constitute about 20% of the total proteins in milk and are generated as by products from cheese manufacture. Regarding ALIBIRD component, a WPC enriched in β-lactoglobulin was hydrolysed under optimized conditions to obtain lactostatin (β-lactoglobulin 71-75, IIAEK), a peptide with hypocholesterolemic activity proved in a rat model (*27, 37*). Other fragments from β-lactoglobulin that were identified in the hydrolysate have been described as bioactive peptides, for instance, IPAVF and VLVLDTDYK with antimicrobial activity (*28*), or ALPMHIR and GLDIQK with angiotensin-I-converting enzyme (ACE)-inhibitory activity (*26, 31*). Among other ACE inhibitors identified in the hydrolysate, peptide YLLF has been also described as opioid (*34*) and VAGTWY has recently been shown to inhibit dipeptidyl peptidase-4 (*35*). Therefore, the hydrolysate could present a preventive effect for lifestyle disease patients such as those with hypertension, hypercholesterolemia or type 2 diabetic mellitus and it can improve gastrointestinal protection.

Other component employed in the formulation of ALIBIRD product is a supercritical extract of rosemary (*Rosmarinus officinalis*) herb. Rosemary is an aromatic plant from Lamiaceae family, typical of the Mediterranean region, which has been recognized to have numerous and important biological properties, such as hepatoprotective (*1*) antidiabetic (*4*), antioxidant (*3, 13*), antiproliferative (*15*), antiviral (*3*), antimicrobial (*5*), and antidepressant (*22*), among others. Nevertheless, the most appreciated and reported property of rosemary is its antioxidant capacity, which is related to the presence of antioxidant phenolic substances, such as carnosol, rosmanol, carnosic acid, methyl carnosate, rosmarinic and caffeic acids (*17, 36*). Moreover, carnosic acid and carnosol are the most abundant antioxidant compounds present in rosemary plant. Some *in vitro* investigations have shown that carnosic acid has an

antioxidant activity three times higher than that of carnosol (*17*). Nevertheless, also the contrary conclusion was reported, depending on the method employed to produce the rosemary extract and to evaluate its antioxidant activity (*29*). In this respect, different authors (*12, 32*) compared rosemary extracts produced by supercritical fluid extraction with those obtained using solid-liquid extraction with different solvents (ethanol and hexane) or hydro-distillation, and demonstrated the superior antioxidant activity of the supercritical extracts.

The idea of combining the supercritical fluid extract of rosemary, hydrolysate of WPC and oligosaccharides derived from lactulose (OsLu) was to obtain a new functional ingredient, ALIBIRD, which may have the health benefits of its components, i.e. able to add multiple functionality to the food providing a wide range of potential benefits without appreciable risk to health. Although the combined ingredients are produced using non-toxic processes to date there have no conventional safety studies published for the components of ALIBIRD, excepting an acute oral safety study of rosemary extracts (*2*). Spurred by the interest in the potential use of ALIBIRD as a dietary supplement for potential chemopreventive effects and to establish toxicological safety data of this product, this research was undertaken to test the acute and subchronic (limit doses) oral toxicity of ALIBIRD, a mixture of oligosaccharides derived from lactulose, hydrolysate of WPC and supercritical fluid rosemary extract in rats, in an experimental design in accordance with the European Union Guidelines (*19, 20*) and under Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

MATERIALS AND METHODS

Test substance. The test substance ALIBIRD is formed by three components: oligosaccharides derived from lactulose, a hydrolysate of WPC enriched in β-lactoglobulin

and rosemary extract in a proportion 1:0.5:0.05. The Table 1 shows the detailed composition of the ALIBIRD product that was employed in the present toxicity studies.

Characterization of ALIBIRD product. Oligosaccharides derived from lactulose (OsLu) were synthesized using a commercial lactulose preparation (Duphalac®) (670 g of lactulose/L) and β-galactosidase from *Aspergillus oryzae* (16 Ud/mL) (Sigma). Enzymatic reactions were carried out at 50 ºC and pH 6.5 in an orbital shaker at 300 rpm for 24 hours. Afterward, samples were immediately immersed in boiling water for 10 min to inactivate the enzyme. Later, the mixture of oligosaccharides was treated with yeasts to eliminate monosaccharides following the method previously described (*33*) with some changes. Briefly, oligosaccharide reaction mixture 20% (w/v) was treated with fresh *Saccharomyces cerevisiae* 1.5 % (w/v) (Levital) at 30ºC for 48h in an orbital shaker (300 rpm) and submitted to vacuum filtration (Whatman nº 1) to remove the yeasts. Sample was dried at 38-40º C in a rotary evaporator (Büchi Labortechnik AG. Flawil, Switzerland). Mono- and disaccharides as well as oligosaccharides derived from lactulose (OsLu) were analysed by GC in form of trimethyl silylated oximes (TMSO) following the method of Montilla et al., (2011) (*25*). GC analyses were performed in an Agilent Technologies 7890A gas chromatograph equipped with a flame ionization detector (FID), using nitrogen as carrier gas at a flow rate of 1 mL/min. The TMSO derivatives, prepared as described Brobst and Lott (1966) (*6*), were separated using a ZB-5HT Inferno^{TM} fused silica capillary column (15 m long x 0.25 mm i.d. x 0.10 μm film thickness) coated with 5% phenyl 95% dimethylpolysiloxane (Phenomenex, Torrance, CA, USA). The initial oven temperature was 150 ºC and raised to 250 ºC at a heating rate of 10 ºC/min, raised again to 380 ºC at 3 ºC/min, and remaining at this temperature for 7 min. The injector (split ratio 1:40) and detector temperatures were 280 ºC and 380 ºC, respectively.

Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software (Wilmington, DE). The identification of TMSO derivatives of carbohydrates

was carried out by comparing the experimental retention indices with those of standards previously derivatized. Quantitative data (g/100 g total carbohydrates) were calculated from FID peak areas. Standard solutions of fructose, galactose, lactulose, kestose and nystose (all of them from Sigma Chemical Co.) over the expected concentration range in samples (0.01-2 mg/mL) were prepared to calculate the response factor relative to phenyl-β-D-glucoside (internal standard). Response factor of kestose and nystose were applied for quantitation of trisaccharides and oligosaccharides with DP ≥ 4, respectively.

Degree of polymerisation (DP) of the oligosaccharides found in the purified fraction from mixture of synthesis was determined by Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by laser desorption were introduced into a time of flight analyser (1.3 m flight path) with an acceleration voltage of 25 kV, 94% grid voltage, 0.025% ion guide wire voltage, and a delay time of 200 ns in the reflector positive ion mode. Mass spectra were obtained over the *m/z* range 100-3000. External mass calibration was applied using the monoisotopic $[M + H]^+$ values of des-Arg^1 bradykinin and angiotensin I of the Calibration Mixture 1, Sequazyme Peptide Mass Standards Kit; Applied Biosystems. 2,5-dihydroxybenzoic acid (>98%, Fluka) at 10 mg/mL in water was used as matrix. Sample was diluted 100 times in water, and mixed with the matrix at a ratio of 1:4 (v:v). One μL of this solution was spotted onto a flat stainless-steel sample plate and dried in air before analysis.

The hydrolysis of WPC was prepared as following: a bovine WPC rich in β-lactoglobulin (Friesland Foods Domo, Zwolle, The Netherlands) was dissolved in water 5% (w/v) and heated at 90 ºC for 10 min at pilot scale. Hydrolysis was carried out by food-grade porcine trypsin (Biocatalysts, Nantgarw, Wales, UK) at 37 ºC and pH 8.0 by addition of 1 M

NaOH (food grade, Aditio, Panreac Química, S.A.U., Castellar del Vallès, Spain) for 3 h with constant agitation. Porcine trypsin was added at the enzyme-to-substrate ratio of 1:20 (w/w). Reactions were stopped by heating at 95 ºC for 15 min, assuring the complete inactivation of the enzyme. The hydrolysate was dried by spray drying. Protein content of the dried hydrolysate was determined by Kjeldahl. RP-HPLC-MS/MS characterization of the hydrolysate was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionization source as previously described (*16*). The column used was a reverse phase XBridge PST C18 Column (150×2.1 mm i.d. 5 μm particle size) (Waters Corp. Milford, MA, USA). The signal threshold to perform tandem mass spectrometry was 50000. The acquired MS/MS spectra were interpreted using BioTools (version 3.1; Bruker Daloniks).

The supercritical extract of rosemary, third component of ALIBIRD product, was performed as following: rosemary (*Rosmarinus officinalis L.*) leaves were ground in a cooled mill and sieved to 200-600 μm. Then, supercritical fluid extraction of the sample was accomplished using a pilot-plant extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell, a two-step decompression system (separators cells S1 and S2) and a CO_2 recirculation device. The extractor and separator cells have independent control of temperature (± 2°C) and pressure (± 0.1 MPa). In the CO_2 recirculation device CO_2 is condensed (≈ 5 MPa), pumped up to the desired extraction pressure and heated up to the selected extraction temperature. According to previous studies (*21*), extraction was carried out for 6 h at 30 MPa and 313 K. In order to remove the essential oil present in the plant matrix and further concentrate the rosemary antioxidant components, fractionation of the extract was carried out during the first hour of extraction (*36*). Then, two

different extracts were collected in S1 and S2, being the extract employed in this work the fraction obtained in S1.

The essential oil compounds present in the supercritical fluid extract of rosemary were determined using a GC-2010 (Shimadzu, Japan), equipped with a split/splitless injector, electronic pressure control, AOC-20i auto injector, GCMS-QP2010 Plus mass spectrometer detector, and a GCMS Solution software. The column used was a ZB-5 (Zebron) capillary column, 30 m x 0.25 mm I.D. and 0.25 μm phase thickness. Helium, 99.996% was used as a carrier gas at a flow of 1 mL/min. Oven temperature programming was 60 ºC isothermal for 4 min then increased to 106 ºC at 2.5 ºC/min and from 106ºC to 130ºC at 1ºC/min and finally from 130ºC to 250 ºC at 20 ºC/min, this temperature was kept constant for 10.2 min. Sample injections (1 μL) were performed in split mode (1:20). The inlet pressure of the carrier gas was 57.5 KPa. Injector temperature was of 250 ºC and MS ion source and interface temperatures were 230 and 280 ºC, respectively. The mass spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu. The quantification of essential oil compounds given in Table 1 was made by means of their corresponding calibration curves.

The content of phenolic compounds was carried out by HPLC analysis of the samples using a HPLC (Varian Pro-star) equipped with a Microsorb-100 C18 column (Varian) of 25 cm × 4.6 mm and 5 μm particle size. The mobile phase consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: 0–8 min, 23% A, 8-25 min, 75% A, 25-40 min 75% A and the 40-45 min 23% A . Initial conditions were gained in 5 min. The flow rate was constant at 0.7 ml/min. Injection volume was 20 μL and the detection was accomplished by using a diode array detection system (Varian) storing the signal at a wavelength of 230, 280 and 350 nm. The quantification of carnosic acid and carnosol was made by comparison with calibration curves.

Different batches of the three components of ALIBIRD product were manufactured, and low deviations were obtained in their composition. In the case of the oligosaccharide-rich component the content of total carbohydrates in mixtures of five different batches elaborated was of 76.2 ± 2.3% (3% deviation) of which 36.4 ± 1.1 % were OsLu (3% deviation). For the WPC hydrolysate, the content of protein in three different batches was 72.56 ± 1.64 % (2.3% deviation) determined by Kjeldahl. Deviation in the content of selected peptides was determined using LC-MS and external calibration curves, and was 3.5% on average. Finally, the content of total essential oil in different batches of the rosemary extract produced was 17.2 ± 1.4 % (8.1% deviation), and the deviation in the content of phenolic diterpenes (carnosic acid + carnosol) was 6.4%. The exact composition of the ALIBIRD product used in the toxicity studies is given in Table 1.

Acute and repeated dose (28 days) oral toxicity studies. Wistar male and female rats (Charles River Inc., Marget, Kent, UK) were acclimated for 7 days prior to study initiation with an evaluation of health status. The rats were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2ºC and 50% ± 10% relative humidity) with a 12h light-dark cycle (light from 08.00 to 20.00h). Food (A03 rodent diet, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) and water were available *ad libitum*. The rats were 56-days old at initiation of treatment. Acute and repeated dose (28 days) studies (limit test) were conducted in accordance with European Union guidelines (*19, 20*). Both studies were undertaken in accordance with the ethics requirements and authorized by the Official Ethical Committee of the Complutense University.

In the acute (limit test) study, 24 rats (12 males, 12 females) were distributed into two groups of 6 males and 6 females each. After an overnight fast, each rat received distillate water orally (control group or Group 1), or a single oral dose of 2000 mg /kg body weight of

ALIBIRD (treated group or Group 2). Doses of the test and control articles were administered by oral gavage at a volume of 10 mL/kg body weight (equivalent to 2000 mg/kg body weight) based on the individual animal body weights obtained on the day dosing. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m). At the end of a 14 days observation period, the rats were weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied.

The repeated dose (28 days) (limit test) study was conducted in 48 rats (24 males, 24 females) divided in four groups of 6 males and 6 females each (control group or Group 3; treated group or Group 4; satellite control group or Group 5; and satellite treated group or Group 6). Rats received a daily dose of either distilled water (Groups 3 and 5) or 2000 mg/kg body weight of the ALIBIRD product (Groups 4 and 6) orally once a day over 4 weeks. Doses of the test and control articles were administered by gavage at a volume of 10 mL/kg body weight (equivalent to 2000 mg/kg body weight) based on the individual animal body weights obtained on the day dosing. Animals were dosed at approximately the same time each day (approximately 4-6 h into light cycle). Food but not water was withheld from 4 h before until 2 h after control and test article administration. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). All rats of the Groups 3 and 4 were deprived of food for 18 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on day 29. All animals of the satellite groups (Groups 5 and 6) were kept a further 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects. All rats of the Groups 5 and 6 were deprived of food for 18 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on day 42.

Observations. All animals were observed twice daily for general appearance, behaviour, signs of morbidity and mortality (once before treatment and once daily thereafter). Rats were observed for their general condition and the condition of the skin and fur, eyes,

nose, oral cavity, abdomen and external genitalia, evaluated for respiration rat and palpated for masses. Behavioural parameters checked were abnormal movements (tremor, convulsion and muscular contractions) reactions to handling and behaviour in open field (excitability, responsiveness to touch and to sharp noise), changes in ordinary behaviour (changes in grooming head shaking and gyration), abnormal behaviour (autophagy, backward motion) and aggression. Body weight, body weight gain and food and water consumption were measured daily, and at the end of the observation periods the rats were examined by necropsy, and the weights of the organs recorded.

Clinical test parameters. Blood samples for haematology and clinical chemistry evaluation were collected from the retro-orbital plexus from animals under light anaesthesia induced by CO_2 inhalation after 14 days observation period in the acute oral study and after 28 days of treatment and 14 days of recovery for the repeated dose 28 days safety study. EDTA was used as an anticoagulant for haematology samples and sodium citrate was used as an anticoagulant for clinical chemistry. Food was withheld for approximately 18h before blood collection, and samples were collected early in the working day to reduce biological variation; water was provided *ad libitum*. Clinical pathology parameters (haematological and clinical biochemistry) were evaluated. Most haematology variables were measured with a Coulter/CELL-DYN 3500 whole blood automated analyzer (Abbott, Chicago, ILL). Blood cell smears were observed with an Olympus Microscopy BX41 (Olympus, Tokyo, Japan). Clinical chemistry parameters were evaluated with a spectrophotometer Konelab PRIME 30 (Thermofisher Scientific Inc. Waltham, Mass., USA) and special biochemistry parameters with a clinical chemistry analyzer AU640 (Olympus, Tokyo, Japan). Coagulation parameters were analyzed with a coagulation analyzer Coatron M1 (Teco Medical Instruments, GMBH, Neufahrn, Germany).

Anatomical pathology. All rats were euthanized by CO_2 inhalation and necropsied. The necropsy included a macroscopic examination of the external surface of the body, all orifices, the cranial cavity, the brain and spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal, and pelvic cavities and viscera. Descriptions of all macroscopic abnormalities were recorded. Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate-buffered 4% formaldehyde solution: adrenal glands, brain, heart, ileum, jejunum, caecum, colon, duodenum, rectum, stomach, oesophagus, trachea, kidneys, liver, lungs, pancreas, spleen, skin, testicles with epididymes, ovaries with oviducts, bone marrow, thymus, thyroid and parathyroid glands, seminal vesicles, urinary bladder and uterus. The organ:body weight ratios were calculated. All organ and tissue samples for histopathological examination were processed, embedded in paraffin, cut at an approximate thickness of 2 to 4 µm, and stained with hematoxylin and eosin. Slides of all organs and tissues listed above were collected from all animals of the control and treated groups.

Statistical analysis. All data are expressed as means ± standard error of the mean (SEM) of 6 determinations for each sex (n = 6). Differences between male control and male treated groups as well as between female control and female treated groups were evaluated with a one-way analysis of variance (ANOVA) followed by Dunnett´s test (*18*), and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

ALIBIRD composition. This product was a mixture of oligosaccharides derived from lactulose, WPC hydrolysate and rosemary extract (1:0.5:0.05). Each component was individually characterized and the detailed composition of each component of the ALIBIRD product is detailed in Table 1.

Oligosaccharides derived from lactulose (OsLu) consisted of a mixture of carbohydrates which after purification presented a 76% of sugars which corresponded to 28 % of monosaccharides, 12 % of lactulose; and 36 % of OsLu (17 % of disaccharides; 13 % of trisaccharides, 5 % of tetrasaccharides; 1 % of pentasaccharides) (Table 1, Figure 1). Although MALDI-TOF-MS analysis of OsLu allowed the detection of oligosaccharides with degree polymerization (DP) 6 (m/z 1175.4; 1029.3), DP7 (m/z 1175.4) and DP8 (m/z 1337.2) (Figure 2), they could not be quantified by CG.

The composition of the WPC hydrolysate is shown in Table 1 and consisted in a 74% of protein, 5% of moisture and 7% of ash. At least a 99% of the protein content corresponded to β-lactoglobulin and no traces of α-lactabumin were found by PAGE-SDS or mass spectrometry (data not shown). The WPC hydrolysated also contained lactose (aprox. 11%) and a small percentage of fat. The peptide composition of the hydrolysate was studied by HPLC-MS/MS and the identified peptides are listed in Fiure 3.

Figure 4 and Figure 5 show the chromatograms obtained in the characterization of rosemary supercritical extract (third component of ALIBIRD product). Figure 4 corresponds to the GC-MS analysis of the volatile oil; the peaks marked in the figure indicate the major compounds identified. The antioxidant phenolic diterpenes, namely carnosic acid and carnosol, were identified and quantified by HPLC according to the method described before and the chromatogram obtained is presented in Figure 5.

Acute oral toxicity in rats. No abnormal clinical signs, behavioural changes, body weight changes, macroscopic findings, or organ weight changes were observed. All animals survived the 2-week observation period. Body weight data are depicted in Figure 6. There were no statistical differences in body weights among groups. Similarly, no statistically significant differences in body weight gain, food and water consumption were noted (data not

shown). Body weight, daily body weight gain, food and water consumption thus were unaffected by the treatment (single oral dose of 2000 mg/kg of the ALIBIRD product).

The haematological and clinical chemistry parameters assessed 2 weeks after administration of the ALIBIRD product as a single oral dose of 2000 mg/kg body weight were not significantly different compared with those of controls (Table 2 and Table 3). Individual values and group mean values were within the physiological range. No treatment-related changes were noted.

There were no statistical differences in organ weight or tissue: body weight ratios related to the test material (data not shown). The ALBIRD product was not associated with any incidence of macroscopic and microscopic changes. No treatment-related histopathological changes were observed 2 weeks after administration of the ALIBIRD product as a single oral dose of 2000 mg/kg body weight, and histological correlates for the organ weight changes were found. Therefore, ALIBIRD has a low order of acute toxicity and that the oral lethal dose (LD_{50}) for male and female rats is higher than of 2000 mg/kg body weight.

Repeat dose (28 days) oral toxicity in rats. No mortality was observed. No treatment related changes in the general condition and external appearance were observed in male and female rats treated with the ALIBIRD product at the 2000 mg/kg body weight daily dose. The development of the animals during the experimental period corresponded to their specie and age.

Body weight and food consumption. There were no significant differences in body weight (data not shown) or body weight gain (Figure 7 and Figure 8) among groups treated with ALIBIRD in comparison to the control group at any time point of the experimental period. All ALIBIRD treated groups consumed similar amounts of food and water (data not shown) to those of the corresponding control groups.

Clinical pathology. All haematological data were within normal limits, and differences between groups were not observed (Table 4). Clinical chemistry data showed no treatment-related alterations at the end of 28days treatment period (Table 5). Individual values and group mean values were within the physiological range. After 14 days without treatment to detect delayed occurrence of potential toxic effects, there were no treatment related changes in haematological and clinical test parameters (data shown in Table 4 and Table 5, satellite treated group).

Anatomical pathology. The necropsy performed on day 29 after the last dose of ALIBIRD (Group 4) and on day 42 after 14 days without any treatment (Group 6) did not reveal any gross pathological changes or any differences in organ weights in comparison to the corresponding control groups. Mean organ weights and rate body weight and organ are presented in Table 6. After 28 days of treatment, there were no histopathological findings in the organs examined considered being treatment related in male and female rats (photomicrographs of tissues not shown; the histological evaluation of all organs studied of the ALIBIRD group demonstrated normal architecture). There were also no treatment related histopathological findings in the satellite treated group (Group 6) (photomicrographs of tissues not shown).

This study represents the first standard toxicological data on the ALIBIRD, product containing oligosaccharides derived from lactulose, a WPC hydrolysate and a rosemary extract. The present study shows that a single and daily oral limit dose of 2000 mg ALIBIRD/ kg body weight (4 weeks) was well tolerated by both male and female rats and there were no evidence for systemic toxicity. A single dose of 2000 mg/kg body weight did not result in any observable adverse effects or mortality. Daily oral administration of 2000 mg/kg body weight (28 days) did not cause mortality, or changes in body weight, body weight gain of food consumption. No haematological or clinical pathologic alterations were noted. Both gross and

histopathological examinations did not reveal any treatment related changes. The no-observed-adverse-effect level in this subchronic toxicity study was the dose tested, i.e. 2000 mg/kg. This finding implies achievement of an applied dosage exaggeration over a potential supplement level in human as well as our results provides the basis for the selection of doses for use in chronic toxicity studies (91 days) if it could be necessary before its use as a dietary supplement in humans.

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FIGURE LEGENDS

FIGURE 1. *GC-profile of TMSO (trimethylsilyl oxime) derivatives of oligosaccharides derived from lactulose (Duphalac®) found on purified mixtures of synthesis. Fr: fructose; Ga: galactose, I.S. internal standard (phenyl-β-D-glucoside); Lu: lactulose; DP: degree of polymerization; DP2: disaccharides; DP3: trisaccharides; DP4: tetrasaccharides; DP5: pentasaccharides.*

FIGURE 2. *MALDI-TOF-MS profile of the purified fraction of oligosaccharides formed during enzymatic hydrolysis of Duphalac® with β-galactosidase from Aspergillus oryzae. DP: degree of polymerization.*

FIGURE 3. *Peptide characterization of the whey protein concentrate (WPC) hydrolysate performed by HPLC and tandem mass spectrometry. Total ion current chromatogram is represented and the identified β-lactoglobulin peptides are indicated.*

FIGURE 4. *GC-MS profile obtained in the analysis of the volatile oil compounds present in rosemary supercritical extract.*

FIGURE 5. *HPLC chromatogram obtained in the analysis of the main antioxidant compounds of rosemary supercritical extract.*

FIGURE 6. *Body weight of rats during the 2-week observation period, following a single oral dose of ALIBIRD at 2000 mg/kg body weight. Mean values, n = 6 animals/sex group, with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.*

FIGURE 7. *Daily body weight gain of rats exposed to repeated (28 days) oral doses of control (◊) and ALIBIRD (□) at 2000 mg/kg body weight. Mean values, n = 6 animals/sex group, with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.*

FIGURE 8. *Daily body weight gain of rats exposed to repeated (28 days) oral doses of control (♦) and ALIBIRD (■) at 2000 mg/kg body weight and moreover observed 14 days after treatment (Satellite Groups). Mean values, n = 6 animals/sex group, with bars equal to the SEM. Absence of bars means that the SEM was less than the size of the symbol.*

TABLE 1. *Composition of three ingredients of ALIBIRD product*

COMPOSITION AND CONTENT OF OLIGOSACCHARIDES DERIVED FROM LACTULOSE (OsLu)	
Carbohydrates (Total 76%)	(%)
Monosaccharides	28
Lactulose	12
Oligosaccharides derived from lactulose: Disaccharides (17%); Trisaccharides (13%); Tetrasaccharides (5%); Pentasaccharides (1%)	36
Moisture	18
Nitrogen	1
Salts	5

COMPOSITION OF THE WHEY PROTEIN CONCENTRATE (WPC) HYDROLYSATE	
Protein	74.07 (%)
Moisture	5.05 (%)
Ash	7.14 (%)
Calcium	0.92 (mg/g)
Magnesium	0.23 (mg/g)
Sodium	16.79 (mg/g)
Potassium	7.72 (mg/g)

COMPOSITION AND CONTENT OF THE ROSEMARY SUPERCRITICAL EXTRACT	
Essential Oil (GC/MS)	
1,8-cineol (eucalyptol)	8.76 (%)
Camphor	3.26 (%)
Borneol,	0.79 (%)
α-Terpineol	0.95 (%)
Verbenone	0.56 (%)
β-Caryophyllene	0.22 (%)
Linalool	0.24 (%)
Bornyl Acetate	0.20 (%)
Rest of compounds (monoterpenes)	3.04 (%)
Phenolic Diterpenes (HPLC)	
Carnosic acid	16.90 (%)
Carnosol	1.93 (%)

TABLE 2. *Haematological parameters in rats after the 2-week observation period following a single oral dose of ALIBIRD at 2000 mg/kg body weight*

Parameters	Acute oral dose			
	Group 1: control		Group 2: treated with ALIBIRD product	
	Females	Males	Females	Males
RBC ($x10^6/\mu l$)	8.04 ± 0.14	8.07 ± 0.21	7.90 ± 0.11	7.95 ± 0.12
Haemoglobin (g/dl)	15.30 ± 0.22	15.35 ± 0.40	14.78 ± 0.18	14.85 ± 0.16
Haematocrit (%)	43.70 ± 0.50	43.98 ± 1.12	42.52 ± 0.54	43.27 ± 0.43
MCV (fl)	52.33 ± 1.20	54.48 ± 0.51	53.80 ± 0.62	54.43 ± 0.40
MCH (pg)	18.32 ± 0.38	19.02 ± 0.13	18.70 ± 0.20	18.68 ± 0.12
MCHC (g/dl)	35.02 ± 0.19	34.55 ± 0.22	34.77 ± 0.19	34.33 ± 0.11
RDW (%)	17.62 ± 0.65	16.98 ± 0.55	16.45 ± 0.50	16.58 ± 0.62
WBC ($x10^3/\mu l$)	4.33 ± 0.25	4.81 ± 0.36	4.03 ± 0.33	5.47 ± 0.51
Banded neutrophils ($x10^3/\mu l$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Neutrophils ($x10^3/\mu l$)	0.79 ± 0.08	1.13 ± 0.10	0.61 ± 0.07	0.91 ± 0.05
Eosinophils ($x10^3/\mu l$)	0.05 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.07 ± 0.01
Lymphocytes ($x10^3/\mu l$)	3.36 ± 0.21	4.53 ± 0.24	3.29 ± 0.34	4.40 ± 0.53
Monocytes ($x10^3/\mu l$)	0.14 ± 0.02	0.16 ± 0.03	0.09 ± 0.01	0.10 ± 0.02
Basophils ($x10^3/\mu l$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets ($x10^3/\mu l$)	818.17 ± 88.88	951.33 ± 48.35	739.97 ± 149.04	875.00 ± 37.36
MPV (fl)	8.37 ± 0.21	7.68 ± 0.47	7.93 ± 0.09	8.08 ± 0.06

Results are expressed as mean ± SEM (n = 6) in each sex group.

Differences between the treated group and the control group were not significant.

TABLE 3. *Clinical chemistry parameters in rats after the 2-week observation period following a single oral dose of ALIBIRD at 2000 mg/kg body weight.*

Parameters	Acute oral dose			
	Group 1: control		Group 2: treated with ALIBIRD product	
	Females	Males	Females	Males
Glucose (mg/dl)	119.33 ± 5.98	135.33 ± 8.23	123.17 ± 2.39	139.50 ± 5.68
Urea nitrogen (mg/dl)	37.33 ± 3.76	36.33 ± 1.05	42.83 ± 1.64	40.50 ± 1.88
Creatinine (mg/dl)	0.53 ± 0.03	0.45 ± 0.02	0.57 ± 0.02	0.48 ± 0.02
Total protein (g/dl)	7.33 ± 0.20	6.27 ± 0.16	7.23 ± 0.12	6.50 ± 0.06
Total bilirubin (mg/dl)	0.18 ± 0.03	0.25 ± 0.07	0.23 ± 0.02	0.22 ± 0.02
Calcium (mg/dl)	11.86 ± 0.17	11.84 ± 0.22	11.83 ± 0.08	12.04 ± 0.13
Sodium (mEq/l)	142.33 ± 1.36	139.83 ± 1.01	140.33 ± 1.98	138.67 ± 0.49
Potassium (mEq/l)	5.95 ± 0.34	6.03 ± 0.31	5.35 ± 0.08	5.85 ± 0.12
ASAT (u/l)	134.17 ± 7.68	136.50 ± 3.00	138.67 ± 6.10	130.00 ± 3.32
ALAT (u/l)	57.33 ± 18.61	45.00 ± 3.30	38.83 ± 1.99	54.50 ± 2.95
Alkaline phosphatase (u/l)	219.33 ± 24.22	594.67 ± 47.72	230.50 ± 23.55	588.17 ± 60.14
Triglyceride (mg/dl)	124.67 ± 20.20	144.00 ± 16.84	170.67 ± 11.02	132.50 ± 10.61
Cholesterol (mg/dl)	64.00 ± 1.63	70.33 ± 4.62	67.00 ± 4.93	63.67 ± 2.36
HDL (mg/dl)	37.30 ± 0.99	40.47 ± 2.52	39.43 ± 2.86	37.20 ± 0.99
LDL (mg/dl)	9.20 ± 1.45	10.20 ± 0.97	6.87 ± 1.14	7.77 ± 1.28

Results are expressed as mean ± SEM (n = 6) in each sex group.

Differences between the treated group and the control group were not significant.

TABLE 4. *Haematological parameters in rats after repeated (28 days) oral dose of ALIBIRD at 2000 mg/kg body weight per day*

Parameters[a]	Group 3: control		Group 4: ALIBIRD		Group 5: satellite control		Group 6: satellite ALIBIRD	
	Females	Males	Females	Males	Females	Males	Females	Males
RBC ($x10^6/\mu l$)	8.64 ± 0.10	8.85 ± 0.12	8.41 ± 0.15	8.94 ± 0.12	8.61 ± 0.10	8.96 ± 0.11	8.51 ± 0.11	9.20 ± 0.12
Haemoglobin (g/dl)	16.35 ± 0.16	16.42 ± 0.11	15.98 ± 0.14	16.48 ± 0.22	16.08 ± 0.18	16.72 ± 0.06	15.97 ± 0.19	16.55 ± 0.16
Haematocrit (%)	47.77 ± 0.46	47.68 ± 0.23	46.33 ± 0.61	47.83 ± 0.76	46.43 ± 0.51	47.30 ± 0.15	45.90 ± 0.76	47.41 ± 0.43
MCV (fl)	54.87 ± 0.39	53.73 ± 0.63	55.00 ± 0.77	53.72 ± 0.58	53.37 ± 0.06	52.77 ± 0.71	53.70 ± 0.81	51.35 ± 0.31
MCH (pg)	18.82 ± 0.03	18.45 ± 0.15	18.92 ± 0.28	18.48 ± 0.16	18.62 ± 0.03	18.73 ± 0.34	18.73 ± 0.25	17.90 ± 0.14
MCHC (g/dl)	34.22 ± 0.23	34.15 ± 0.19	34.42 ± 0.06	34.47 ± 0.14	34.88 ± 0.08	35.33 ± 0.15	34.98 ± 0.14	34.88 ± 0.10
RDW (%)	17.97 ± 0.25	19.00 ± 0.35	17.68 ± 0.50	18.82 ± 0.33	16.90 ± 0.76	18.55 ± 0.15	16.77 ± 0.50	19.22 ± 0.22
WBC ($x10^3/\mu l$)	7.05 ± 0.43	8.81 ± 0.33	7.80 ± 0.33	9.72 ± 0.72	7.95 ± 0.50	7.93 ± 0.57	6.80 ± 0.55	8.09 ± 0.65
Banded neutrophils ($x10^3/\mu l$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Neutrophils ($x10^3/\mu l$)	1.50 ± 0.07	2.11 ± 0.17	1.65 ± 0.07	2.26 ± 0.18	1.64 ± 0.10	1.85 ± 0.14	1.43 ± 0.09	1.89 ± 0.13
Eosinophils ($x10^3/\mu l$)	0.05 ± 0.02	0.08 ± 0.03	0.07 ± 0.03	0.08 ± 0.03	0.10 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.11 ± 0.01
Lymphocytes ($x10^3/\mu l$)	5.31 ± 0.38	6.49 ± 0.26	5.61 ± 0.23	7.27 ± 0.56	4.87 ± 0.28	6.00 ± 0.45	4.02 ± 0.33	6.00 ± 0.56
Monocytes ($x10^3/\mu l$)	0.10 ± 0.01	0.11 ± 0.01	0.08 ± 0.02	0.18 ± 0.04	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Basophils ($x10^3/\mu l$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets ($x10^3/\mu l$)	873.83 ± 24.79	856.67 ± 24.69	933.83 ± 34.74	939.50 ± 30.48	862.00 ± 7.16	866.83 ± 47.01	898.00 ± 45.77	969.33 ± 22.07
MPV (fl)	8.13 ± 0.12	8.35 ± 0.06	8.35 ± 0.08	8.08 ± 0.14	8.28 ± 0.07	8.35 ± 0.04	8.12 ± 0.05	8.15 ± 0.07

Results are expressed as mean ± SEM (n = 6) in each sex group.
Differences between ALIBIRD group and control group or ALIBIRD satellite group and satellite control group were not significant.
[a] RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, nucleated red blood cell count; WBC, white blood cell count; MPV, mean platelet volume.

TABLE 5. *Clinical chemistry in rats after repeated (28 days) oral dose of ALIBIRD at 2000 mg/kg body weight per day*

Parameters[a]	Group 3: control		Group 4: ALIBIRD		Group 5: satellite control		Group 6: satellite ALIBIRD	
	Females	Males	Females	Males	Females	Males	Females	Males
Glucose (mg/dl)	118.67 ± 2.38	115.33 ± 2.63	102.83 ± 7.44	111.00 ± 4.43	121.83 ± 4.24	111.67 ± 3.67	107.67 ± 5.49	98.17 ± 3.76
Urea nitrogen (mg/dl)	39.83 ± 1.40	35.50 ± 0.76	37.00 ± 0.68	35.67 ± 0.49	40.50 ± 2.58	37.17 ± 1.01	37.50 ± 0.76	34.50 ± 0.91
Creatinine (mg/dl)	0.58 ± 0.02	0.53 ± 0.02	0.53 ± 0.02	0.52 ± 0.02	0.58 ± 0.02	0.50 ± 0.03	0.55 ± 0.02	0.55 ± 0.02
Albumine (g/dl)	3.80 ± 0.04	3.62 ± 0.03	3.87 ± 0.06	3.58 ± 0.02	3.87 ± 0.09	3.62 ± 0.03	4.07 ± 0.05	3.63 ± 0.03
Total protein (g/dl)	6.95 ± 0.21	6.92 ± 0.05	7.22 ± 0.11	6.73 ± 0.12	7.08 ± 0.22	6.47 ± 0.08	7.43 ± 0.10	6.77 ± 0.10
Total bilirubin (mg/dl)	0.23 ± 0.02	0.22 ± 0.02	0.25 ± 0.02	0.17 ± 0.03	0.18 ± 0.03	0.22 ± 0.03	0.22 ± 0.03	0.25 ± 0.02
Calcium (mg/dl)	10.45 ± 0.06	10.30 ± 0.04	10.42 ± 0.14	10.43 ± 0.05	10.07 ± 0.16	10.21 ± 0.11	10.51 ± 0.17	10.34 ± 0.15
Sodium (mEq/l)	144.67 ± 0.49	143.17 ± 0.40	143.00 ± 0.63	143.50 ± 0.43	144.17 ± 0.60	142.67 ± 0.49	143.67 ± 0.80	143.83 ± 0.62
Potassium (mEq/l)	6.02 ± 0.12	6.37 ± 0.12	6.28 ± 0.31	6.53 ± 0.30	5.72 ± 0.13	6.57 ± 0.14	5.93 ± 0.25	6.33 ± 0.16
ASAT (u/l)	102.17 ± 4.17	129.67 ± 7.16	135.83 ± 14.97	125.33 ± 7.85	108.83 ± 7.39	114.83 ± 7.96	105.33 ± 5.58	137.67 ± 11.59
ALAT (u/l)	38.50 ± 2.28	44.67 ± 0.92	41.50 ± 2.01	41.17 ± 1.28	27.00 ± 0.58	39.50 ± 0.89	29.33 ± 1.58	37.50 ± 1.43
Alkaline phosphatase (u/l)	230.67 ± 21.54	499.67 ± 28.11	184.50 ± 14.59	472.67 ± 28.46	147.50 ± 6.03	392.33 ± 25.76	154.17 ± 12.64	398.33 ± 22.84
Triglyceride (mg/dl)	124.50 ± 11.79	138.33 ± 17.38	141.50 ± 9.79	147.00 ± 19.17	95.83 ± 4.65	171.00 ± 4.53	112.00 ± 6.26	173.83 ± 4.55
Total cholesterol (mg/dl)	77.17 ± 2.77	63.17 ± 1.70	67.50 ± 3.77	57.67 ± 2.03	62.50 ± 5.77	69.17 ± 1.92	67.83 ± 3.85	64.67 ± 3.39
HDL (mg/dl)	47.85 ± 1.01	39.27 ± 0.78	42.92 ± 2.56	37.30 ± 1.59	40.90 ± 2.95	40.90 ± 1.04	40.92 ± 1.98	39.13 ± 1.93
LDL (mg/dl)	8.95 ± 0.86	11.18 ± 3.18	7.38 ± 0.77	7.93 ± 1.96	16.07 ± 1.76	9.45 ± 1.30	11.75 ± 1.13	11.98 ± 2.02
Lipoproteína A	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34	<2.34
Prothrombin time (seg)	23.10 ± 0.23	25.95 ± 0.60	24.17 ± 0.49	25.23 ± 0.55	25.70 ± 0.66	27.92 ± 0.36	26.12 ± 0.21	27.15 ± 0.35
Thromboplastin partial time (seg)	20.87 ± 0.79	15.55 ± 0.37	22.87 ± 0.57	16.40 ± 1.14	18.85 ± 1.15	19.75 ± 0.84	20.22 ± 1.37	20.98 ± 0.70
Fibrinogen (mg/dl)	323.33 ± 24.77	476.17 ± 17.61	265.00 ± 25.94	408.50 ± 28.61	275.50 ± 19.39	322.33 ± 13.35	324.00 ± 14.63	352.50 ± 16.04

Results are expressed as mean ± SEM (n = 6) in each sex group.
Differences between ALIBIRD group and control group or ALIBIRD satellite group and satellite control group were not significant.
[a]ASAT, aspartate amino transferase; ALAT, alanine amino transferase; HDL, high density lipoprotein; LDL, low density lipoprotein

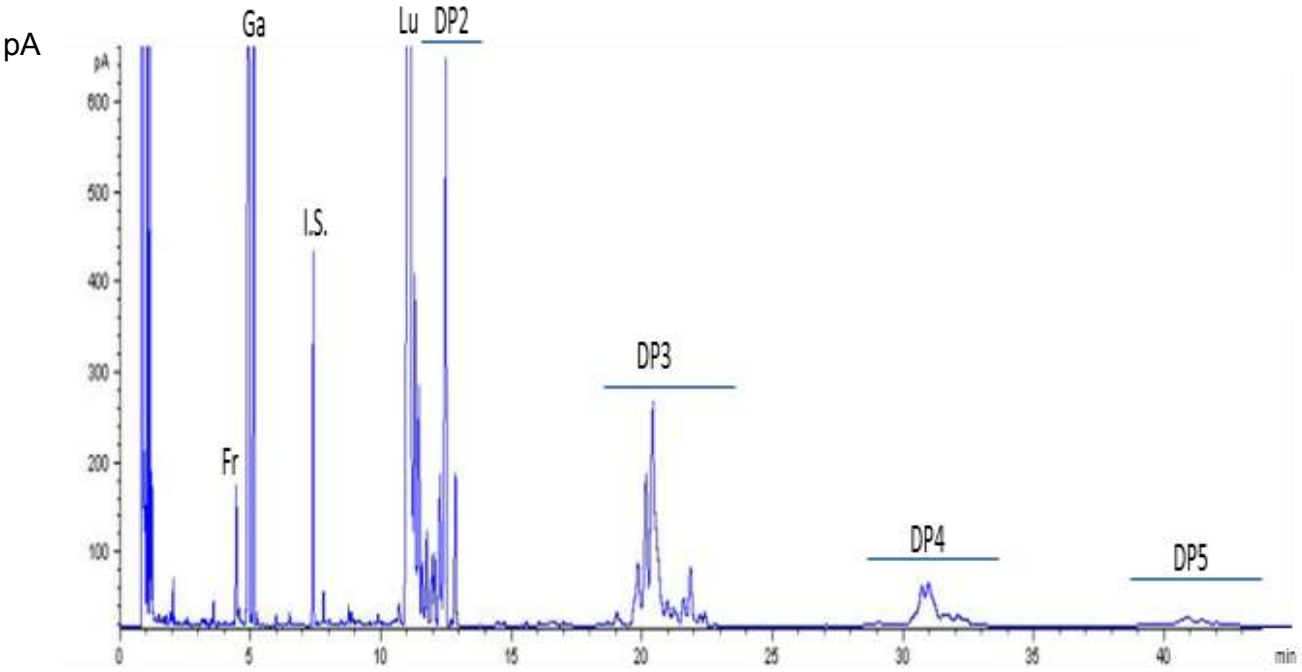
TABLE 6. *Mean organ weights and rate body weight (wt)/organ in rats after repeated (28 days) oral doses of ALIBIRD at 2000 mg/kg body weight per day*

Parameters	Group 3: control		Group 4: ALIBIRD		Group 5: satellite control		Group 6: satellite ALIBIRD	
	Females	Males	Females	Males	Females	Males	Females	Males
Body wt (g)	234.83 ± 0.70	319.50 ± 3.36	234.67 ± 0.92	327.33 ± 4.33	240.67 ± 0.61	346.17 ± 1.22	241.00 ± 1.37	344.83 ± 3.22
Increase body wt (g)	60.50 ± 1.23	117.17 ± 3.24	61.50 ± 1.59	124.50 ± 3.68	79.67 ± 1.58	147.67 ± 1.28	78.00 ± 2.08	146.00 ± 3.44
Brain wt (g)	1.63 ± 0.04	1.92 ± 0.01	1.76 ± 0.05	1.87 ± 0.03	1.82 ± 0.03	1.88 ± 0.01	1.75 ± 0.01	1.87 ± 0.02
Rate body wt/brain (%)	0.693 ± 0.018	0.602 ± 0.007	0.751 ± 0.021	0.572 ± 0.012	0.754 ± 0.014	0.544 ± 0.004	0.726 ± 0.005	0.542 ± 0.008
Thymus wt (g)	0.396 ± 0.069	0.420 ± 0.012	0.448 ± 0.031	0.385 ± 0.047	0.385 ± 0.011	0.457 ± 0.035	0.398 ± 0.006	0.547 ± 0.040
Rate body wt/thymus (%)	0.169 ± 0.029	0.131 ± 0.003	0.191 ± 0.013	0.117 ± 0.013	0.160 ± 0.004	0.132 ± 0.010	0.165 ± 0.003	0.159 ± 0.012
Heart wt (g)	0.609 ± 0.010	0.816 ± 0.013	0.648 ± 0.021	0.869 ± 0.034	0.635 ± 0.019	0.870 ± 0.007	0.623 ± 0.014	0.852 ± 0.011
Rate body wt/heart (%)	0.259 ± 0.005	0.255 ± 0.004	0.276 ± 0.009	0.266 ± 0.010	0.264 ± 0.008	0.251 ± 0.002	0.258 ± 0.005	0.247 ± 0.002
Right lung wt (g)	0.711 ± 0.036	0.741 ± 0.005	0.703 ± 0.024	0.738 ± 0.015	0.582 ± 0.012	0.811 ± 0.028	0.565 ± 0.032	0.776 ± 0.025
Rate body wt/right lung (%)	0.303 ± 0.015	0.232 ± 0.002	0.300 ± 0.010	0.225 ± 0.004	0.242 ± 0.005	0.234 ± 0.008	0.234 ± 0.012	0.225 ± 0.008
Left lung wt (g)	0.378 ± 0.015	0.399 ± 0.015	0.379 ± 0.024	0.383 ± 0.012	0.316 ± 0.004	0.466 ± 0.012	0.317 ± 0.010	0.415 ± 0.024
Rate body wt/left lung (%)	0.161 ± 0.006	0.125 ± 0.004	0.162 ± 0.010	0.117 ± 0.005	0.131 ± 0.002	0.134 ± 0.003	0.131 ± 0.004	0.120 ± 0.007
Liver wt (g)	7.83 ± 0.25	11.11 ± 0.18	8.39 ± 0.06	11.43 ± 0.18	6.61 ± 0.16	10.79 ± 0.18	7.13 ± 0.36	11.00 ± 0.31
Rate body wt/liver (%)	3.33 ± 0.11	3.48 ± 0.06	3.57 ± 0.03	3.49 ± 0.03	2.75 ± 0.07	3.12 ± 0.05	2.95 ± 0.14	3.19 ± 0.08
Spleen wt (g)	0.526 ± 0.029	0.648 ± 0.018	0.522 ± 0.014	0.704 ± 0.029	0.536 ± 0.019	0.649 ± 0.022	0.488 ± 0.029	0.656 ± 0.026
Rate body wt/spleen (%)	0.224 ± 0.012	0.203 ± 0.007	0.222 ± 0.006	0.215 ± 0.009	0.223 ± 0.008	0.188 ± 0.007	0.202 ± 0.012	0.190 ± 0.007
Pancreas wt (g)	0.381 ± 0.056	0.528 ± 0.120	0.472 ± 0.096	0.501 ± 0.033	0.350 ± 0.048	0.367 ± 0.036	0.385 ± 0.107	0.291 ± 0.075
Rate body wt/pancreas (%)	0.162 ± 0.024	0.165 ± 0.038	0.201 ± 0.041	0.153 ± 0.010	0.145 ± 0.020	0.106 ± 0.011	0.160 ± 0.045	0.085 ± 0.022
Right kidney wt (g)	0.665 ± 0.016	0.957 ± 0.012	0.697 ± 0.029	0.979 ± 0.020	0.589 ± 0.024	0.912 ± 0.033	0.634 ± 0.028	0.895 ± 0.014
Rate body wt/right kidney (%)	0.283 ± 0.007	0.300± 0.004	0.297 ± 0.012	0.299 ± 0.008	0.241 ± 0.007	0.263 ± 0.010	0.263 ± 0.011	0.260 ± 0.005
Left kidney wt (g)	0.698 ± 0.021	0.954 ± 0.009	0.733 ± 0.027	0.938 ± 0.034	0.581 ± 0.017	0.868 ± 0.031	0.536 ± 0.099	0.907 ± 0.020
Rate body wt/left kidney (%)	0.297 ± 0.009	0.299 ± 0.005	0.312 ± 0.011	0.287 ± 0.013	0.241 ± 0.007	0.251 ± 0.009	0.222 ± 0.041	0.263 ± 0.006
Right adrenal gland wt (g)	0.0037 ± 0.003	0.025 ± 0.002	0.045 ± 0.003	0.034 ± 0.003	0.043 ± 0.003	0.033 ± 0.003	0.035 ± 0.004	0.034 ± 0.002
Rate body wt/right adrenal gland (%)	0.016 ± 0.001	0.008 ± 0.001	0.019 ± 0.001	0.010 ± 0.001	0.018 ± 0.001	0.010 ± 0.001	0.014 ± 0.002	0.010 ± 0.001
Left adrenal gland wt (g)	0.050 ± 0.005	0.030 ± 0.004	0.050 ± 0.006	0.034 ± 0.004	0.040 ± 0.003	0.030 ± 0.001	0.033 ± 0.002	0.035 ± 0.004
Rate body wt/left adrenal gland (%)	0.021 ± 0.002	0.009 ± 0.001	0.021 ± 0.002	0.010 ± 0.001	0.017 ± 0.001	0.009 ± 0.0004	0.014 ± 0.001	0.010 ± 0.001
Right testicle wt (g)		1.65 ± 0.04		1.63 ± 0.06		1.61 ± 0.06		1.67 ± 0.04
Rate body wt/right testicle (%)		0.516 ± 0.012		0.496 ± 0.014		0.466 ± 0.018		0.484 ± 0.015
Right epididymide wt (g)		0.523 ± 0.042		0.510 ± 0.040		0.560 ± 0.052		0.683 ± 0.139
Rate body wt/right epididymide (%)		0.164 ± 0.014		0.157 ± 0.014		0.162 ± 0.015		0.197 ± 0.039
Left testicle wt (g)		1.59 ± 0.04		1.67 ± 0.04		1.47 ± 0.18		1.73 ± 0.06
Rate body wt/left testicle (%)		0.497 ± 0.011		0.512 ± 0.012		0.424 ± 0.053		0.502 ± 0.017
Left epididymide wt (g)		0.575 ± 0.120		0.504 ± 0.040		0.620 ± 0.053		0.648 ± 0.138
Rate body wt/left epididymide (%)		0.181 ± 0.039		0.155 ± 0.014		0.179 ± 0.015		0.187 ± 0.039
Bone marrow wt (g)	0.073 ± 0.010	0.055 ± 0.008	0.087 ± 0.006	0.066 ± 0.007	0.070 ± 0.012	0.049 ± 0.006	0.078 ± 0.004	0.051 ± 0.004
Rate body wt/bone marrow (%)	0.031 ± 0.004	0.017 ± 0.002	0.037 ± 0.003	0.020 ± 0.002	0.029 ± 0.005	0.014 ± 0.002	0.032 ± 0.002	0.015 ± 0.001

Results are expressed as mean ± SEM (n = 6) in each sex group.

Differences between ALIBIRD group and control group or ALIBIRD satellite group and satellite control group were not significant

Fig.1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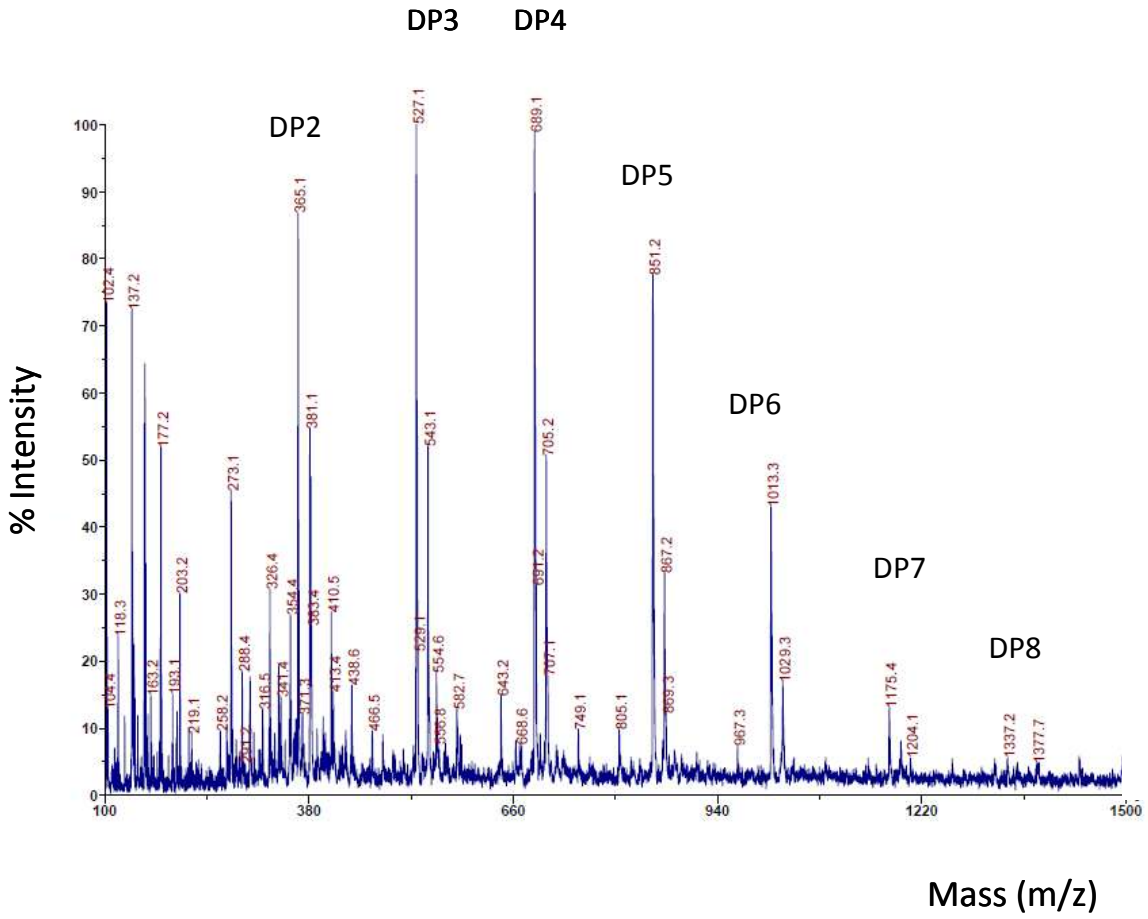
Ga
Lu
DP2
I.S.
Fr
DP3
DP4
DP5
pA
600
500
400
300
200
100
0
5
10
15
20
25
30
35
40
min
time (min)

1

Fig.2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DP4
DP2
DP5
DP6
DP7
DP8
% Intensity
Mass (m/z)
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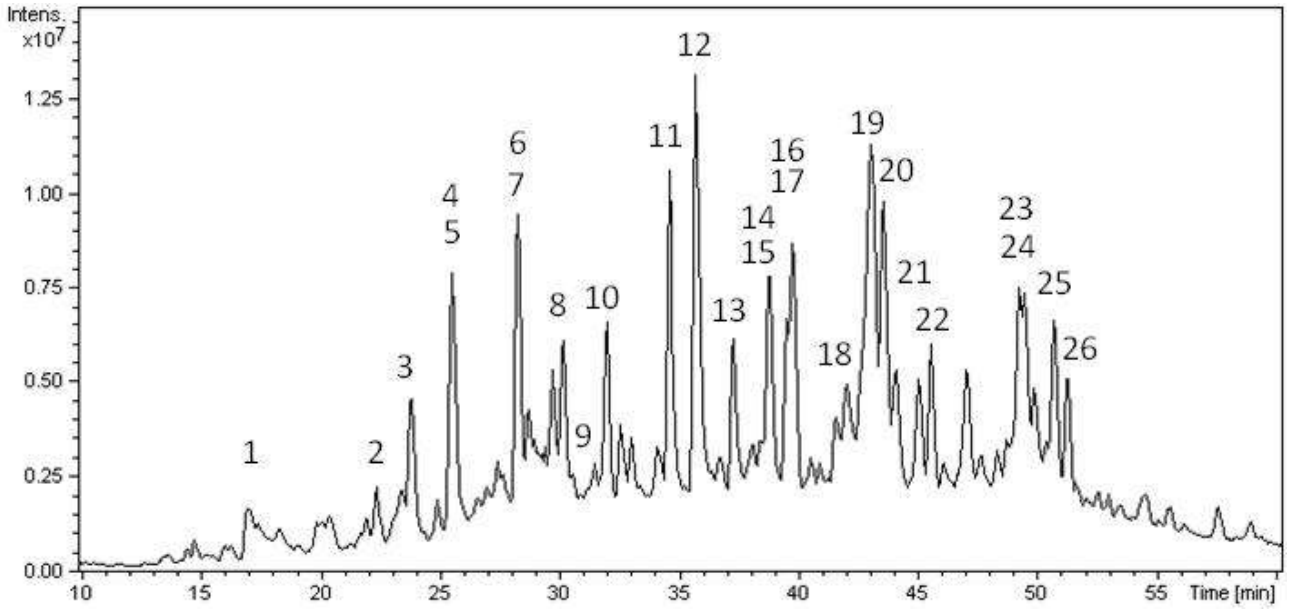
380
660
940
1220
1500

Fig.3



Compound	β-lactoglobulin fragment
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4	70 - 75
5	33 - 40
6	84 - 91
7	83 - 91
8	8 - 14
9	125 - 135
10	1 - 8
11	142 - 148
12	92 - 101
13	76 - 83
14	78 - 83
15	125 - 138
16	15 - 20
17	92 - 100
18	76 - 82
19	78 - 82
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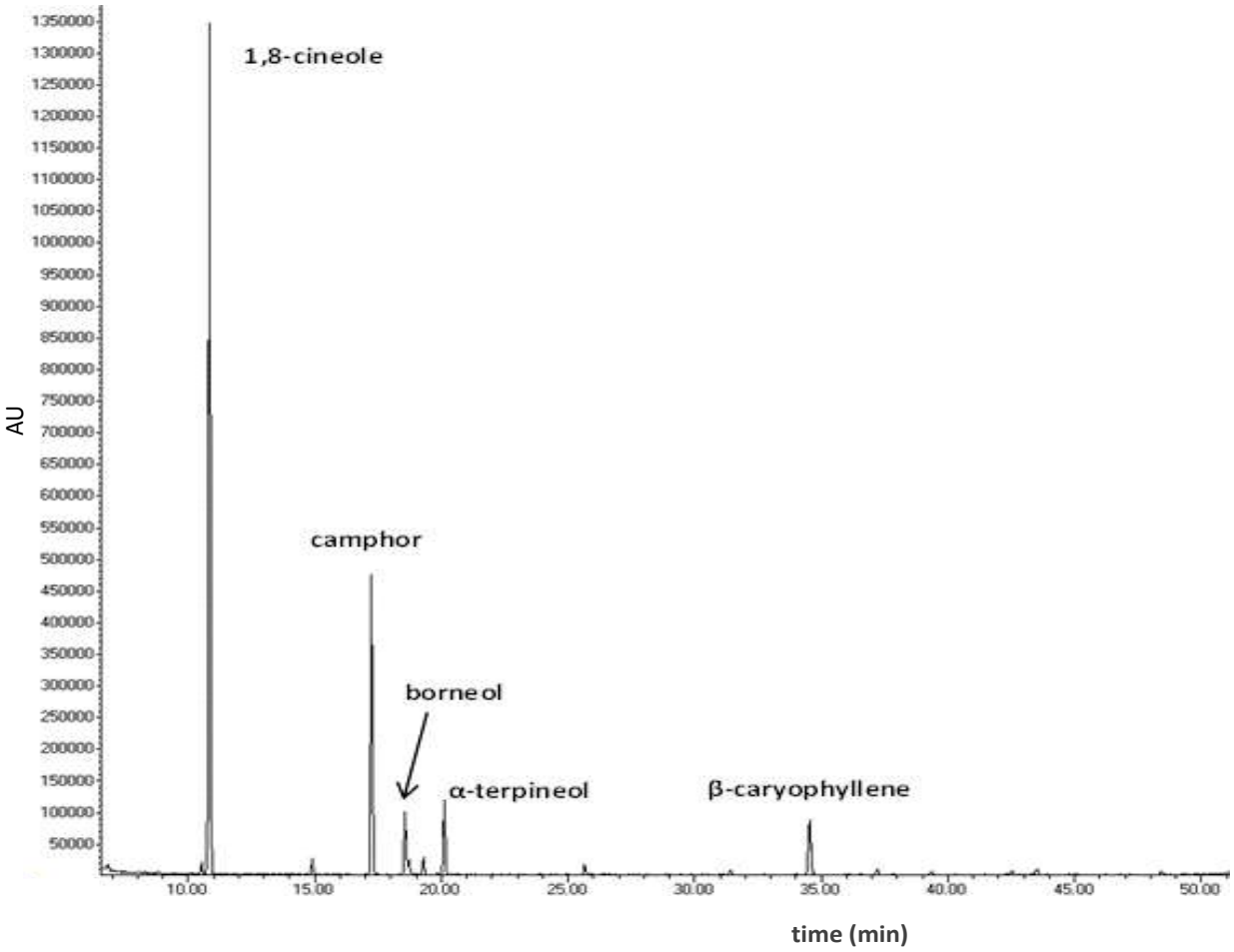
24	52 - 57
25	21 - 32
26	102-105

Fig.4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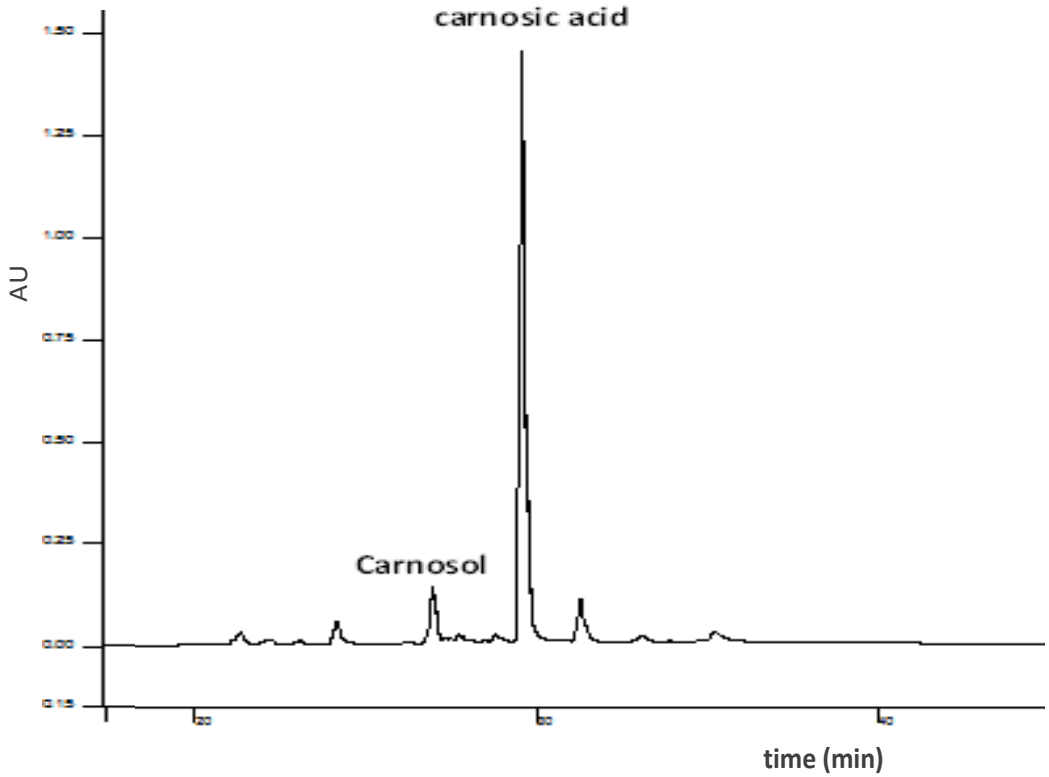
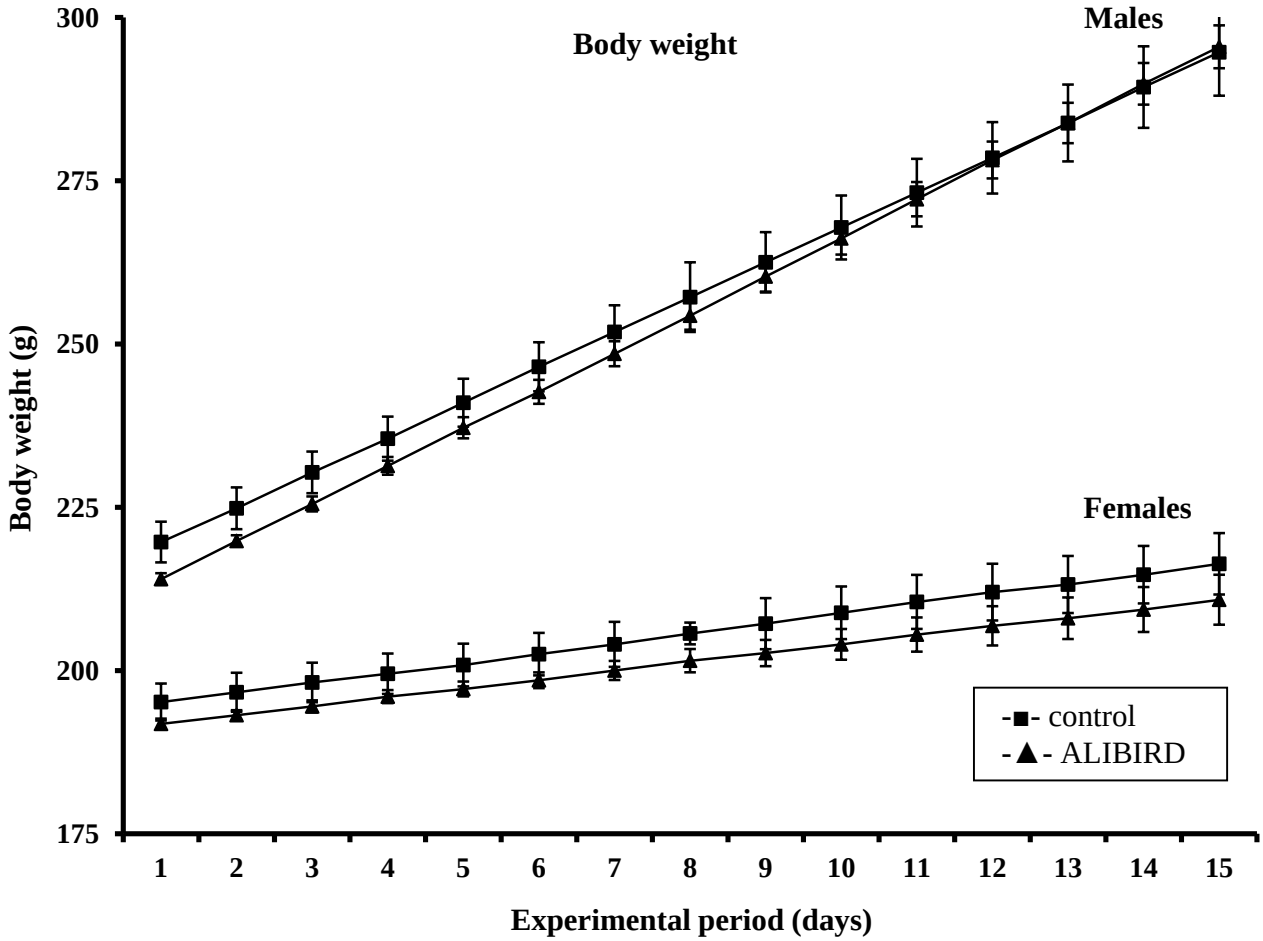
4

Fig.5



5

Fig.6


Body weight
Males
Females
Body weight (g)
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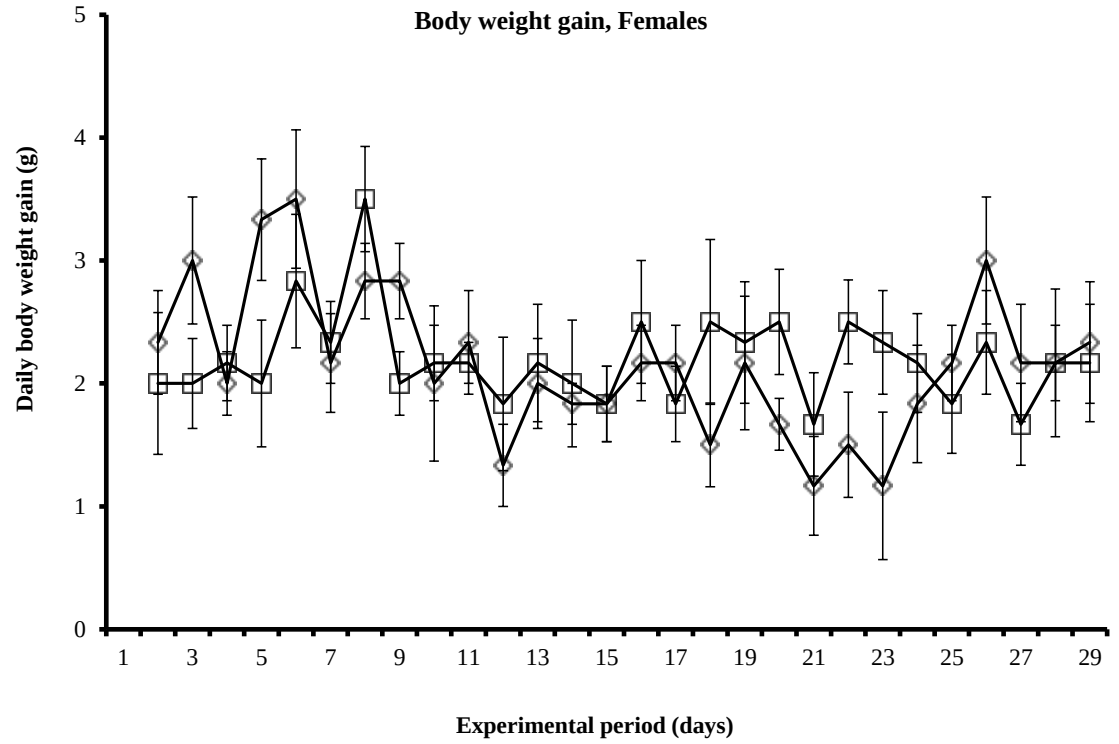
275
250
225
200
175
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Experimental period (days)
-■- control
-▲- ALIBIRD

6

Fig.7


Body weight gain, Females
Daily body weight gain (g)
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7
9
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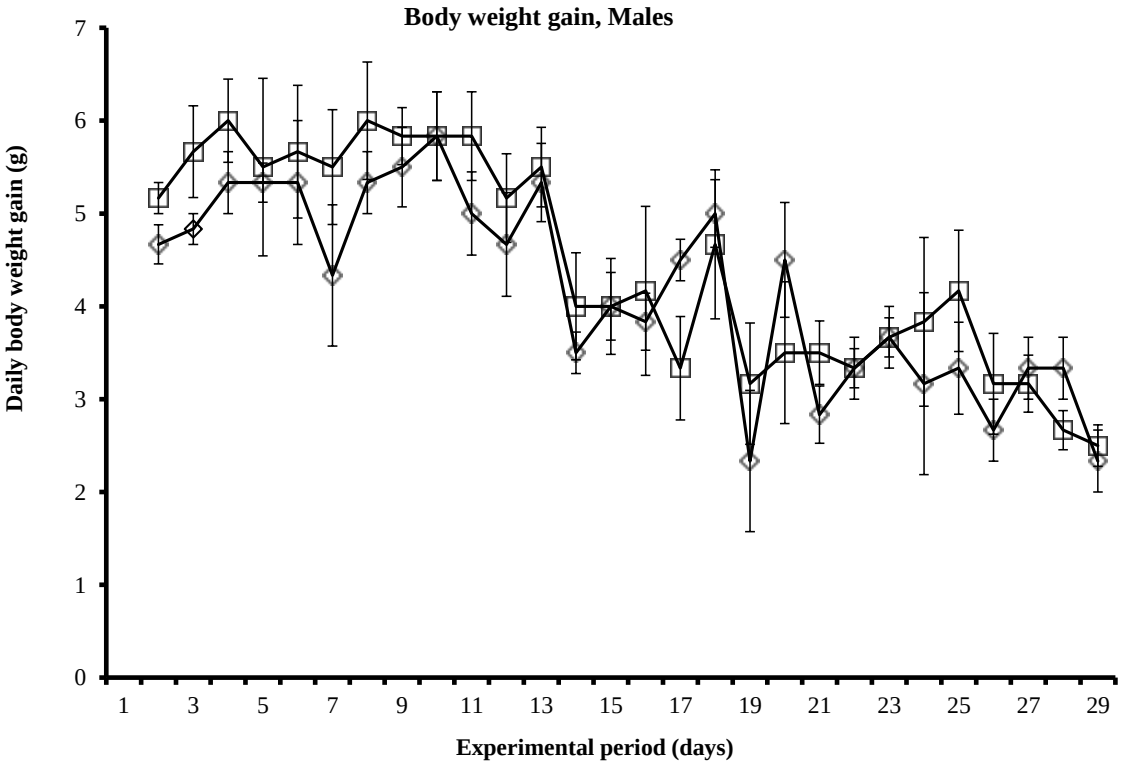
15
17
19
21
23
25
27
29
Experimental period (days)


Body weight gain, Males
Daily body weight gain (g)
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6
7
1
3
5
7
9
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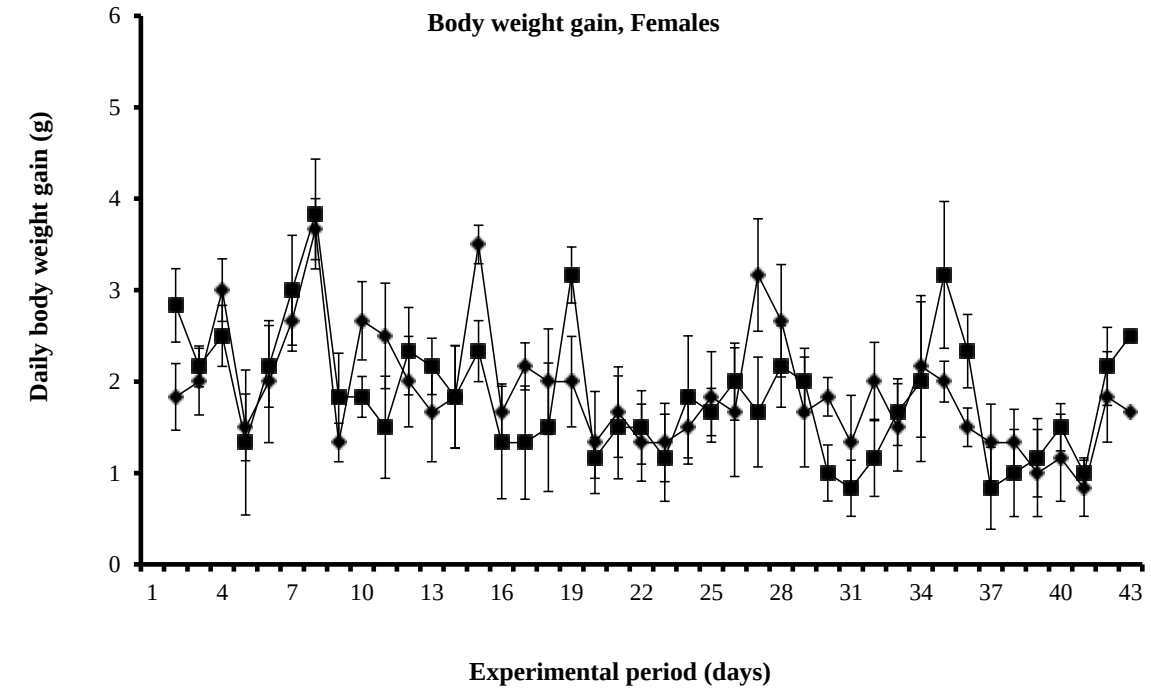
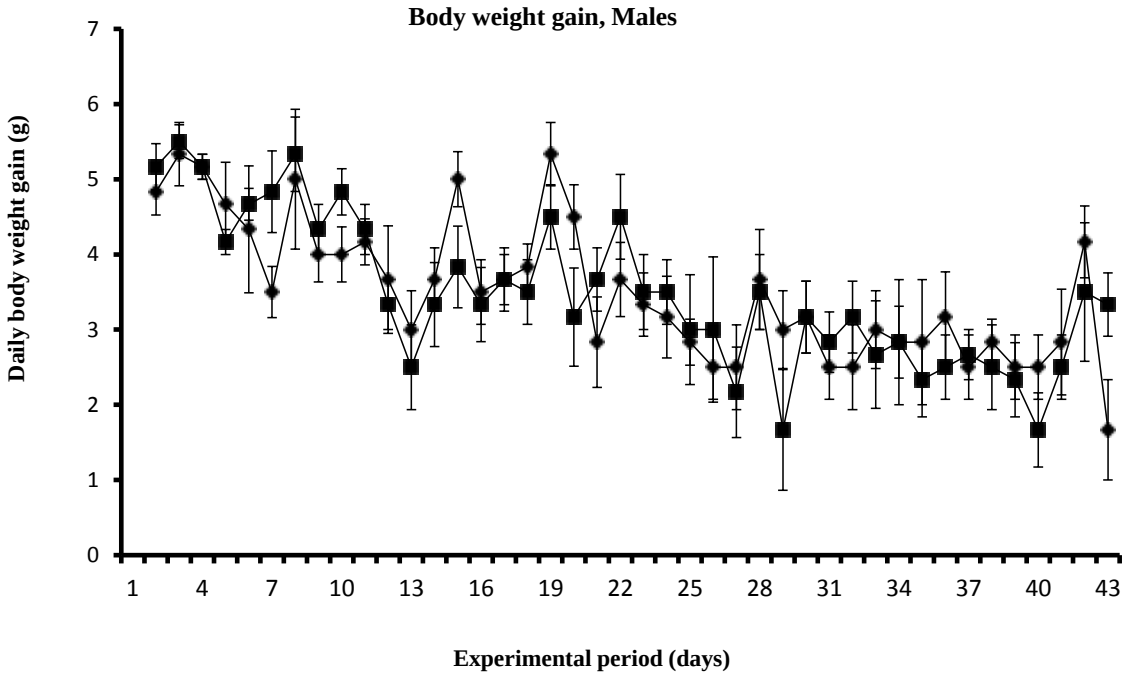
13
15
17
19
21
23
25
27
29
Experimental period (days)

7

Fig.8


Body weight gain, Females
Daily body weight gain (g)
0
1
2
3
4
5
6
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
Experimental period (days)


Body weight gain, Males
Daily body weight gain (g)
0
1
2
3
4
5
6
7
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
Experimental period (days)

8

El final del camino será el principio de otro más largo,

ahora creo que estamos preparados para ser mucho mejores…

Aquellos maravillosos años (LHR)